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Syntheses of subtractively modified 2-chloro-4-nitrophenyl β-maltopentaosides and their application to the differential assay of human alpha-amylases

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ABSTRACT

Three novel maltopentaosides, 2-chloro-4-nitrophenyl *O*-(6-deoxy-α-D-*xylo*-hex-5-enopyranosyl)-(1 → 4)-tris[*O*-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**3**), 2-chloro-4-nitrophenyl *O*-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**10**), and 2-chloro-4-nitrophenyl *O*-(3,6-anhydro-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**26**) were synthesized by chemical and enzymatic reactions. Two human alpha-amylases, salivary alpha-amylase (HSA) and pancreatic alpha-amylase (HPA), hydrolyzed **3** and **10** with the same specificity, almost entirely at a single D-glucosidic linkage, but had no hydrolytic acitivity for **26**. Compound **3** was hydrolyzed by each of these amylases at an approximately equal rate, while **10** was hydrolyzed by HSA 4-fold faster than by HPA. Taking advantage of the difference in the hydrolytic rate of **10**, we developed a new method for the differential assay of these two human alpha-amylases.

INTRODUCTION

Determination of the total activity of alpha-amylase (EC 3.2.1.1) in human serum and urine is of diagnostic value in various diseases[1]. It is also well known that there are two human alpha-amylases, salivary alpha-amylase (HSA) and pancreatic alpha-amylase (HPA). Some diseases (e.g., acute pancreatitis) cause an increase of HPA, and others (e.g., parotitis) cause an increase of HSA. In order to make an accurate diagnosis, the differential assay of the two amylases is very important. Their separation has been carried out by electrophoresis[2], isoelectric

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focusing[3], and chromatography[4], but these methods are time-consuming. Several methods using amylase inhibitor[5,6] have been reported; however, this substance does not inhibit the amylase stoichiometrically[7]. Other methods, based on the difference in the ratio of the products in the reaction of the two amylases with (2-pyridyl)amino derivatives of malto-oligosaccharides[8] or a galactopyranosyl derivative of 4-nitrophenyl α-maltopentaosides[9], have been proposed, but are cumbersome for clinical application, because of the necessity for numerous steps in the HPLC analysis.

Our studies of the influence of various modifications at a terminal (nonreducing-end) D-glucosyl group of maltopentaosides on the alpha-amylase hydrolysis have utilized many systematically synthesized substrates in an attempt to determine a useful substrate for alpha-amylase assay[10,11]. Our finding that the 6^5-deoxy-6^5-fluoro derivative was hydrolyzed by HSA approximately 2-fold faster than by HPA led us to expect that maltopentaosides with subtractive modifications (i.e., dehydration or reduction) at the terminal glucosyl group would be hydrolyzed at a markedly different rate by the two amylases. Such compounds would then be potential substrates for the differential assay.

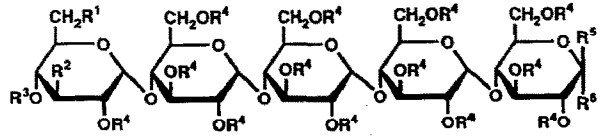
The object of the present work was to synthesize subtractively modified maltopentaosides and to identify a potential substrate for the differential assay. We now describe the synthesis of three novel 2-chloro-4-nitrophenyl β-maltopentaosides, the interesting mode of actions of the two amylases on these compounds, and a new method of differential assay using one of them.

RESULTS AND DISCUSSION

Synthesis.—We selected maltopentaose as the malto-oligosyl moiety and 2-chloro-4-nitrophenol as the aglycon of the synthesized substrates, as in a previous paper[11]. For the purpose of the subtractive modifications, we designed a synthesis for anhydro and deoxy derivatives of maltopentaosides.

The 6^5-deoxy-5^5-eno derivative was first synthesized as follows. Reaction of 2-chloro-4-nitrophenyl *O*-(2,3,4-tri-*O*-acetyl-6-deoxy-6-iodo-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1 → 4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside[11] (**1**) with AgF in pyridine gave 2-chloro-4-nitrophenyl *O*-(2,3,-4-tri-*O*-acetyl-6-deoxy-α-D-*xylo*-hex-5-enopyranosyl)-(1 → 4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1 → 4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**2**) in 79% yield. *O*-Deacetylation of **2** with K_2CO_3 in MeOH gave 2-chloro-4-nitrophenyl *O*-(6-deoxy-α-D-*xylo*-hex-5-enopyranosyl)-(1 → 4)-tris[*O*-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**3**) in 80% yield.

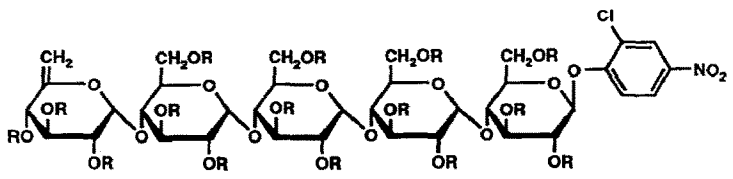
The second target compound, the 6^5-deoxy derivative, was synthesized by two routes. The first route was a chemical synthesis from maltopentaose (**4**) as follows. Benzylidenation of **4** with benzaldehyde dimethyl acetal in *N*,*N*-dimethylformamide (DMF) in the presence of *p*-toluenesulfonic acid, followed by acetylation, gave the 4^5,6^5-*O*-benzylidene derivative **5** in 67% yield from **4**. Then, *O*-(2,3-di-



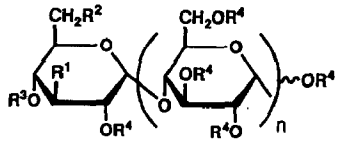
Compound	R^1	R^2	R^3	R^4	R^5	R^6
1	I	OAc	Ac	Ac	CNP	H
8	H	OAc	Bz	Ac	H	Br
9	H	OAc	Bz	Ac	CNP	H
10	H	OH	H	H	CNP	H
26	-O-		H	H	CNP	H
27	OH	OH	H	H	CNP	H

Abbreviations: CNP, 2-chloro-4-nitrophenyl.

O-acetyl-4-*O*-benzoyl-6-bromo-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1 → 4)]-1,2,3,6-tetra-*O*-acetyl-D-glucopyranose (**6**) was prepared in 81% yield by the oxidative bromination of **5** with *N*-bromosuccinimide (NBS) in CCl_4–1,1,2,2-tetrachloroethane. The C–Br bond of **5** was reductively cleaved by hydrogenolysis with Pd–C in 1,4-dioxane to obtain *O*-(2,3-di-*O*-acetyl-4-*O*-benzoyl-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1 → 4)]-1,2,3,6-tetra-*O*-acetyl-D-glucopyranose (**7**) in 89% yield. Attempts to hydrogenolyze **1** or similar 6^5-substituted maltopentaosides[11] having a leaving group by various methods[12] were unsuccessful, because the NO_2 group of the 2-chloro-4-nitrophenyl moiety was reduced before cleavage of the C–X bond at the 6^5-position. Reaction of **7** with PBr_3 in CH_2Cl_2 in the presence of a trace amount of H_2O gave bromide **8** (89%), which was converted by the Koenigs–Knorr method[13] into the glycoside 2-chloro-4-nitrophenyl *O*-(2,3-di-*O*-acetyl-4-*O*-benzoyl-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1 → 4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**9**) in 82% yield. *O*-Deacetylation of **9** with NH_4OH in MeOH–H_2O gave the desired 2-chloro-4-nitrophenyl *O*-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**10**) in 72% yield.



Compound	R
2	Ac
3	H



Compound	R^1	R^2	R^3	R^4	n
4	OH	OH	H	H	4
5	OH	–OCHPh–		H	4
6	OAc	Br	Bz	Ac	4
7	OAc	H	Bz	Ac	4
14	OH	H	H	H	0
15	OH	H	H	H	1
16	OH	H	H	H	2
17	OH	H	H	H	3
18	OH	H	H	H	4
19	OH	H	H	H	5
20	OH	H	H	H	6
21	OAc	H	Ac	Ac	4
23	–O–		H	H	3
24	–O–		H	H	4
25	–O–		H	H	5

The second route was an enzymatic synthesis from cyclomaltohexaose (**11**), using a unique cyclodextrin-hydrolyzing enzyme (CDase)[14] as follows. The selective mono-*O*-*p*-toluenesulfonylation of **11** with 4 equiv of reagent in pyridine at room temperature gave 6-*O*-*p*-toluenesulfonylcyclomaltohexaose (**12**, 29%), and reductive C–OTs bond cleavage with $NaBH_4$ in Me_2SO then gave 6-deoxycyclomaltohexaose (**13**, 76%)[15]. The CDase hydrolyzed **13** at seven positions of α-D-glucosidic bonds and subsequent exo-type hydrolysis by glucoamylase gave 6-deoxy-D-glucose (**14**, 15%) and six malto-oligosaccharides having a 6-deoxy-D-glucosyl group at the nonreducing end: 6^2-deoxymaltose (**15**, 14%), 6^3-deoxymaltotriose (**16**, 4.0%), 6^4-deoxymaltotetraose (**17**, 6.6%), 6^5-deoxymaltopentaose (**18**, 5.3%), 6^6-deoxymaltohexaose (**19**, 4.1%), and 6^7-deoxymaltoheptaose (**20**, 4.2%) (Fig. 1). The CDase hydrolyzed **13** at seven positions, whereas the enzyme hydrolyzed glucosyl- or maltosyl-cyclomaltoheptaose at a single position remote from the 6-*O*-glucosyl- or 6-*O*-maltosyl-glucosyl moiety[16]. It thus seemed to be that the enzyme recognized the 6-*O*-deoxyglucosyl moiety as a simple glucosyl moiety. Of the six deoxy-oligosaccharides, **18** was acetylated in the usual manner to give *O*-(2,3,4-tri-*O*-acetyl-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris-[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1 → 4)]-1,2,3,6-tetra-*O*-acetyl-D-glucopyranose (**21**) in 92% yield. Then, 1-bromination, glycosylation, and subsequent *O*-deacetylation, in a manner similar to the conversion of **7** into **10**, gave the desired **10** in 52% yield from **21**.

The third target compound was the $3^5,6^5$-anhydro derivative. Attempts to construct the 3,6-anhydroglucosyl moiety using 6^5-deoxy-6^5-iodo (**1**), 6^5-bromo-6^5-deoxy, and 6^5-*O*-Ts derivatives[11] as reactive starting materials by reported meth-

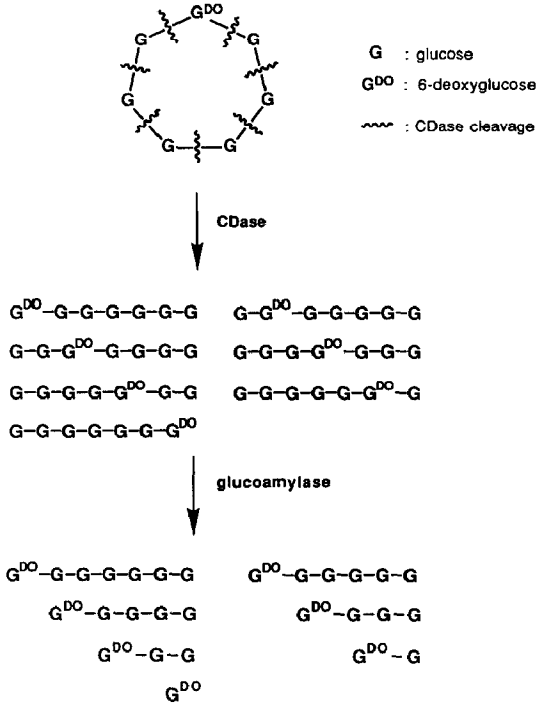
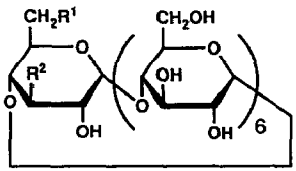


Fig. 1. Schematic representation of enzymatic synthesis of malto-oligosaccharides having a 6-deoxy-glucosyl group at the nonreducing end.

ods[17,18] were unsuccessful, because of the instability of the 2-chloro-4-nitrophenyl β-D-glucosidic bond under such strong basic conditions. Therefore, the $3^5,6^5$-anhydro derivative was synthesized by an enzymatic method from cyclomaltoheptaose in a way similar to that for **21**. Treatment of **12** with KOH in MeOH–H_2O gave 3,6-anhydrocyclomaltohexaose[18] (**22**, 82%). Compound **22** was cleaved by the CDase and subsequent exo-type hydrolysis by glucoamylase afforded three malto-oligosaccharides having a 3,6-anhydroglucosyl group at the nonreducing end:



Compound	R^1	R^2
11	OH	OH
12	OTs	OH
13	H	OH
22	–O–	

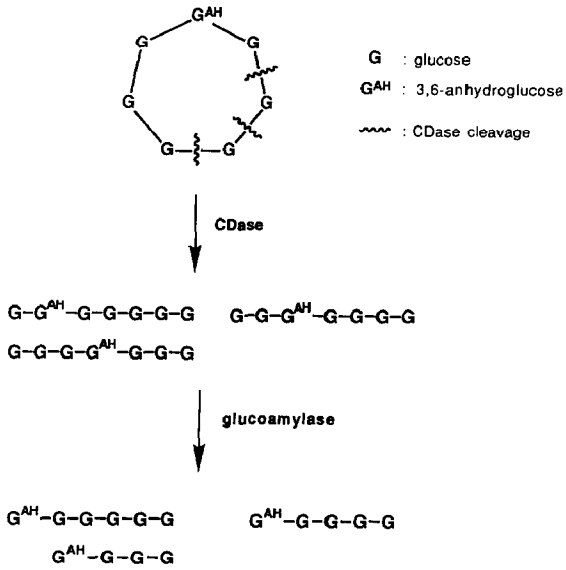


Fig. 2. Schematic representation of enzymatic synthesis of malto-oligosaccharides having a 3,6-anhydro-glucosyl group at the nonreducing end.

$3^4,6^4$-anhydromaltotetraose (**23**, 11%), $3^5,6^5$-anhydromaltopentaose (**24**, 34%), and $3^6,6^6$-anhydromaltohexaose (**25**, 20%) (Fig. 2). It is noteworthy that this case was different from that of **13** or branched cyclomaltoheptaose[16], since the CDase hydrolyzed at three positions away from the 3,6-anhydroglucosyl moiety. Then, *O*-acetylation, 1-bromination, glycosylation, and subsequent *O*-deacetylation in a manner similar to the conversion of **20** into **10** afforded the desired 2-chloro-4-nitrophenyl *O*-(3,6-anhydro-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**26**) in 41% yield from **24**.

Confirmation of structures of maltopentaosides.—The structures of **3**, **10**, and **26** were established by spectral data and elemental analyses as described in the Experimental section. The ^{1}H NMR spectra of the three compounds each showed a signal (1 H, doublet) at δ 5.2–5.3 assigned to H-1a and having large coupling constants ($J_{1a,2a}$ 7.0–7.5 Hz), and also a characteristic signal pattern for the 2-chloro-4-nitrophenyl group in the aromatic area, indicating the presence of a 2-chloro-4-nitrophenyl β-glucoside structure. It also showed signals (4 H, each a doublet) at δ 5.0–5.3 assigned to H-1b–1e having small coupling constants ($J_{1b-e,2b-e}$ 3.0–4.5 Hz), indicating the presence of four α-glucosidic bonds. These results revealed that the three compounds had a 2-chloro-4-nitrophenyl β-maltopentaoside structure. Additionally, the ^{1}H NMR spectrum of **3** showed two doublet signals (1 proton each, *J* 1.8 Hz) at δ 4.56 and 4.71 due to the methylene protons in a 1,1-disubstituted ethylene group, suggesting the presence of the 6-deoxy-α-D-*xylo*-hex-5-enopyranosyl moiety. The ^{1}H NMR spectrum of **10** showed a doublet signal (3 protons, *J* 5.9 Hz) at δ 1.28 due to the methyl protons in a

TABLE I

Kinetic parameters for the action of two human alpha-amylases on maltopentaosides

Compound	K_m (mM)		Relative rate of hydrolysis		
	HPA	HSA	HPA	HSA	V_{HPA} / V_{HSA}
3	0.13	0.23	0.81	0.68	1.19
10	0.025	0.083	0.125	0.50	0.25
26 [a]			0	0	
27	0.29	0.37	1.00 [b]	1.00 [b]	1.00

[a] Both of the human alpha-amylases hardly hydrolyzed **26**. [b] The relative rates of hydrolysis assume the value of unity for **27**.

1,1-disubstituted ethane group, suggesting the presence of the 6-deoxyglucopyranosyl moiety. The ^{13}C NMR spectrum of **26** showed the presence of a unique methylene carbon (δ 68.4) as well as the normal methylene carbon ($-CH_2OH$, δ 60–61). The chemical shift of the unique carbon was very close to that of the methylene carbon (C-6) in methyl 3,6-anhydro-α-D-glucopyranoside[19] (δ 68.2), suggesting that **26** had the 3,6-anhydro-bridged structure.

Kinetic parameters and patterns of action of two human alpha-amylases on modified maltopentaosides.—Maltopentaosides (**3**, **10**, and **26**) were tested to determine the kinetic parameters and the patterns of action of HPA and HSA. In the evaluation of kinetic parameters, each sample was incubated with each of the two alpha-amylases in the presence of coupled enzymes (α- and β-D-glucosidases) in phosphate buffer (pH 7.0) at 37°C, which is the optimum pH and temperature for the human alpha-amylase reaction[20], as described in the Experimental section. The Michaelis constant (K_m) and the relative rate of hydrolysis of the maltopentaosides were examined by incubation, and the amount of products was measured by the increments of absorbance per min at 400 nm. The K_m values were calculated by the method of least squares using Lineweaver–Burk plots. In the study of patterns of action, each sample was incubated with each of the two amylases in the absence of coupled enzymes, and aliquots were analyzed by HPLC in order to determine the amounts of the hydrolyzed products. These results are shown in Tables I and II.

TABLE II

Patterns of action of two human alpha-amylases on substrate-modified maltopentaosides

Compound	Ratios of products with HPA				Ratios of products with HSA			
	G_4-CNP:	G_3-CNP:	G_2-CNP:	G_1-CNP [a]	G_4-CNP:	G_3-CNP:	G_2-CNP:	G_1-CNP
3		0.02:	0.97:	0.01		0.02:	0.97:	0.01
10			0.98:	0.02			0.99:	0.01

[a] Abbreviations: G_4-CNP, 2-chloro-4-nitrophenyl β-maltotetraoside; G_3-CNP, 2-chloro-4-nitrophenyl β-maltotrioside; G_2-CNP, 2-chloro-4-nitrophenyl β-maltoside; and G_1-CNP, 2-chloro-4-nitrophenyl β-D-glucopyranoside.

Compd. No.	Active site		Frequency	
	S_9 S_8 S_7 S_6	S_5 S_4 S_3 S_2 S_1	HPA	HSA
3	BG - G-	G - G - G - CNP	0.02	0.02
	BG - G - G-	G - G - CNP	0.96	0.97
	BG - G - G - G-	G - CNP	0.01	0.01
10	BG - G - G-	G - G - CNP	0.98	0.99
	BG - G - G - G-	G - CNP	0.02	0.01

Fig. 3. Schematic representation of substrate binding to subsites of two human alpha-amylases. Abbreviations: BG, modified glucosyl moiety; G, glucosyl moiety; CNP, β-2-chloro-4-nitrophenyl moiety.

The active sites of depolymerases and especially of such endoglycanases as human alpha-amylase are believed to be tandem subsites geometrically complementary to several glucose residues[21]. As a matter of convenience, it is assumed that the active site of the enzyme includes nine subsites per glucosyl moiety (G) numbered S_1–S_9 from the reducing end, and that glucosidic bonds of the substrates are split between S_5 and S_6 as shown in Fig. 3. The binding modes of the substrates to the active sites during the course of hydrolysis can therefore be estimated from the action patterns of the enzymes on the substrates.

In contrast to our assumption, Table I indicated that there was not a simple tendency; subtractive modification made at the 6^5-position of maltopentaosides did not necessarily afford a low ratio of the relative rate of hydrolysis by the two amylases (V_{HPA}/V_{HSA}). Three compounds of similar subtractive modification displayed remarkably different characteristics for the two amylases. For the 5^5-eno derivative (**3**), the two amylases hydrolyzed the substrate with a high degree of specificity at a single D-glucosidic linkage, to give the same products at an approximately equal rate, which was similar to the case of maltopentaosides having a hydrophobic group at 6-position of their terminal glucosyl moiety[11]. This finding suggested that both active sites of the two amylases recognized **3** in roughly the same manner.

With the 6^5-deoxy derivative (**10**), the most important finding was that the substrate was hydrolyzed by HSA 4-fold faster than by HPA. Taking advantage of this difference in hydrolytic rate, a new method for the differential assay was developed as described below. Additionally, the binding modes of **10** were like that of **3** or hydrophobically modified derivatives, but K_m values for the former were markedly smaller than those for the latter, suggesting that both amylases had high affinity for **10**, especially in the case of HPA. Surprisingly, for the third derivative (**26**), the enzymes could not catalyze the hydrolysis, so it was considered that the

substrate was incorporated to form a nonproductive ES-complex. These findings, obtained using three subtractive modified derivatives, provided interesting information for further study of the active site in alpha-amylases and assay of the enzyme.

Application of **10** *to the differential assay of HPA and HSA.*—When a sample containing HPA and/or HSA hydrolyzes both substrate **10** and an unmodified maltopentaoside (**27**)[22], equations for the straight line of the increments of absorbance are:

$$A_1 = k_1 \cdot a_p + k_2 \cdot a_s \quad (1)$$

$$A_2 = k \cdot (a_p + a_s) \quad (2)$$

where A_1 and A_2 are, respectively, the increase in absorbance at 400 nm per min (ΔA) for the enzymatic reaction with **10** and **27** as a substrate, a_p and a_s are HPA and HSA activities (U/L) in a sample, k_1 and k_2 are rate constants of the hydrolysis of **10** by HPA and HSA, and k is the rate constant of the hydrolysis of **27** by each of the amylases. Since $k_1 = 0.125 \cdot k$ and $k_2 = 0.5 \cdot k$, as shown in Table I, eqs *1* and *2* can be transformed to:

$$a_p = (4A_2 - 8A_1)/3k \quad (3)$$

$$a_s = (8A_1 - A_2)/3k \quad (4)$$

Since k is the constant determined by the assay procedure used, eqs *3* and *4* indicate that only two measurable amounts (A_1 and A_2) can give the value of a_p and a_s, that is, the differential assay itself. When 1 unit (U) is the activity of the enzyme which hydrolyzes 1 μmol of **27** per min at 37°C and ϵ of 2-chloro-4-nitrophenol is 16100 (ref. 23), $k = 1.24 \times 10^{-3}$ ($L \cdot \Delta A/U$) under the conditions described in the Experimental section.

We first tested the accuracy of the method for the differential assay. Activities of the commercially available standard HPA and HSA were determined by the usual method using **27** (ref. 24) as the substrate, and samples containing various known activities of each amylase were prepared. Each sample was incubated with **10** or **27** as the substrate in the presence of coupled enzymes in phosphate buffer (pH 7.0) at 37°C as described. From the amounts of A_1 and A_2 obtained, values of a_p and a_s were calculated using eqs *3* and *4*. These results are summarized in Figs 4 and 5, both of which indicated highly linear relationships, thus demonstrating the accuracy of the method. Secondly, we examined the usefulness of the method for the differential assay. Values of a_p in sera from 50 healthy persons were calculated in the same way as described; data were compared by the differential assay method (enzyme immunoassay method) using monoclonal antibodies of HSA (Fig. 6). There is a clear interrelation between our method and the enzyme immunoassay method.

Therefore, we concluded that this new differential assay method, which is the first one based on the difference in the modes of action of the two amylases using

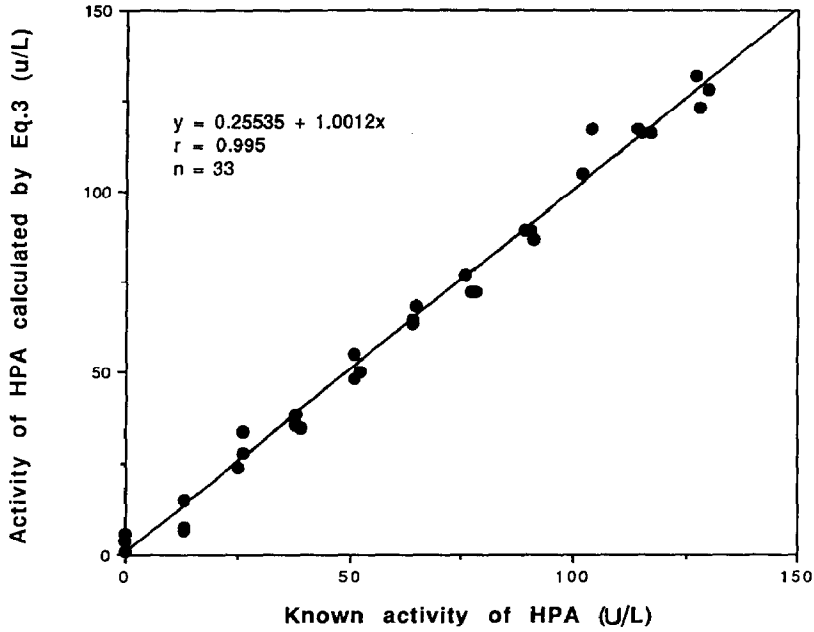


Fig. 4. The relation between known activity and activity calculated by eq *3* of HPA in prepared samples.

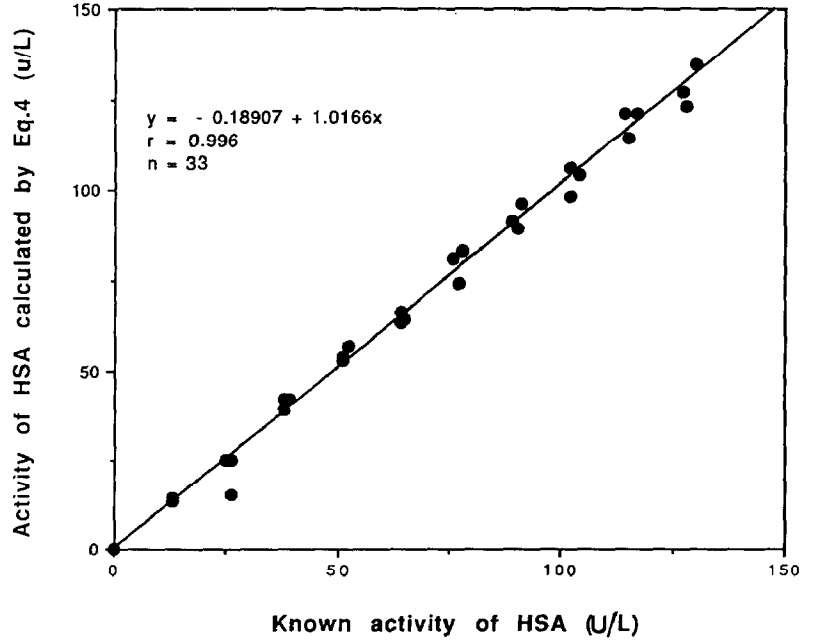


Fig. 5. The relation between known activity and activity calculated by eq *4* of HSA in prepared samples.

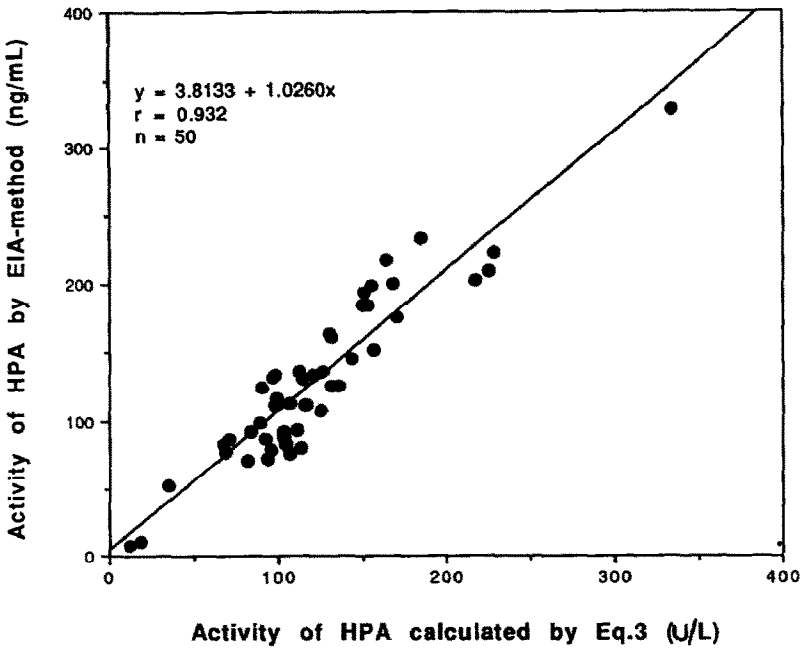


Fig. 6. Correlation of our method with the EIA method for determination of HPA in human sera.

10 and **27** as substrates, is applicable to the measurement of HPA and HSA in human serum. The method has been shown to be convenient, accurate, and very suitable for routine clinical use.

EXPERIMENTAL

Reagents and materials.—All chemicals were of reagent grade unless otherwise noted. Alpha-amylases (from human pancreatic juice and saliva) were obtained from International Reagents Corp., Japan. α-D-Glucosidase (from yeast), β-D-glucosidase (from sweet almond), and glucoamylase (from *Rizopus* sp.) were obtained from Toyobo Co., Ltd., Japan. Maltopentaose (**4**) was obtained from Wako Pure Chemical Industries, Ltd., Japan. The enzyme immunoassay-kit of HPA (RIGHT ASSAY 'P-AMYLASE') was obtained from Sanko Junyaku Co., Ltd., Japan.

Apparatus.—All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were determined with a JASCO DIP-360 digital polarimeter at 25°C. IR spectra were recorded with a JASCO A-202 spectrometer. ^{1}H NMR spectra were recorded at 199.5 MHz and ^{13}C NMR spectra at 50.10 MHz with a JEOL JNM-FX200 spectrometer and Me_4Si as internal standard. ^{1}H and ^{13}NMR spectra were recorded in solutions of 10:1 Me_2SO-d_6–D_2O, unless stated otherwise. High-performance liquid chro-

matography (HPLC) was performed on *A*, a COSMOSIL C_{18} column (4.6 mm i.d. × 150 mm); or *B*, a TSK gel Amide-80 column (4.6 mm i.d. × 250 mm); with a flow rate of 1.0 mL/min using a JASCO pump (880-PU), and a UV (280 nm) detector (JASCO UVIDEC-100-V) or ri detector (Shodex RI SE-51) at room temperature. Visible spectra (400 nm) were recorded with a Hitachi M-80 spectrometer. Column chromatography was performed on Merck Kieselgel 60 (SiO_2, 230–400 mesh) and YMC-GEL ODS-AQ (120-S50, from Yamamura Chemical Laboratories Co., Ltd., Japan).

2-Chloro-4-nitrophenyl O-*(2,3,4-tri-*O*-acetyl-6-deoxy-α-*D*-xylo-hex-5-enopyranosyl)-(1 → 4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-*D*-glucopyranosyl)-(1 → 4)]-2,3,6-tri-*O*-acetyl-β-*D*-glucopyranoside* (**2**).—Silver fluoride (5.56 g, 43.8 mmol) and dimethylaminopyridine (80.0 mg, 0.656 mmol) were added to a solution of 2-chloro-4-nitrophenyl *O*-(2,3,4-tri-*O*-acetyl-6-deoxy-6-iodo-α-D-glucopyranosyl)-(1 → 4)-tris[*O*-(2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl)-(1 → 4)]-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside[11] (**1**; 7.50 g, 4.35 mmol) in pyridine (450 mL) containing 4A molecular sieves (15.0 g). The mixture was stirred for 20 h at room temperature, and inorganic material was collected on a layer of Celite and washed with CH_2Cl_2. The combined filtrate and washings were evaporated in vacuo to leave a syrupy residue, which was chromatographed on SiO_2 gel with 33:1:100 EtOAc–MeOH–CH_2Cl_2 to give **2** (6.19 g, 3.88 mmol, 78.6%); mp 121–123°C (from Et_2O); $[\alpha]_D$ +80.9° (*c* 0.52, 1,4-dioxane); ν_{max} 3480 (OH), 2960 (CH, aliph.), 1752 (C=O), 1664 (C=C), 1586, 1486 (arom.), 1528, 1370 (NO_2), 1430, 1350, 1234, and 1034 (C–O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.99–2.34 (cluster of s, 45 H, 15 OAc), 3.80–4.55 (m, 18 H, H-4a–e,5a–e,6a–d), 4.68–4.95 (5 H, m, H-2a–e), 5.15–5.55 (12 H, m, H-1a–e,3a–e,6e), 7.28 (d, 1 H, *J* 9.0 Hz, H-6 of CNP), 8.15 (dd, 1 H, *J* 9.0 and 2.7 Hz, H-5 of CNP), and 8.29 (d, 1 H, *J* 2.7 Hz, H-3 of CNP); t_R (column *A*; eluent: 3:1 CH_3CN–H_2O): 10.2 min. Anal. Calcd for $C_{66}H_{82}ClNO_{42}$: C, 49.64; H, 5.18; N, 0.88. Found: C, 49.92; H, 5.02; N, 0.77.

2-Chloro-4-nitrophenyl O-*(6-deoxy-α-*D*-xylo-hex-5-enopyranosyl)-(1 → 4)-tris[*O*-(α-D-glucopyranosyl)-(1 → 4)]-β-D-glucopyranoside* (**3**).—Potassium carbonate (285 mg, 2.07 mmol) was added to a suspension of **2** (3.00 g, 1.88 mmol) in MeOH (300 mL) with stirring, and the reaction mixture was kept at room temperature for 16 h. Then, 100 mM phosphate buffer (pH 6.5, 300 mL) was added to the solution, half of the solvent was evaporated in vacuo, and a solution of the residue in H_2O was obtained. ODS gel column chromatography (H_2O → 5% → 10% → 20% CH_3CN, stepwise) of the solution gave **3** (1.45 g, 1.50 mmol, 79.8%); pale-yellow, amorphous; $[\alpha]_D$ +75.9° (*c* 0.52, H_2O); ν_{max} 3420 (OH), 2940 (CH, aliph.), 1656 (C=C), 1584, 1484 (arom.), 1520, 1350 (NO_2), 1276, 1150, and 1026 (C–O) cm^{-1}; ^1H NMR: δ 3.05–3.85 (m, 27 H, H-2a–e to H-4a–e, H-5a–d, and H-6a–d), 4.56 and 4.71 (d, each 1 H, *J* 1.8 Hz, H-6e), 5.08 (d, 2 H, *J* 3.2 Hz, H-1), 5.15 (d, 1 H, *J* 3.9 Hz, H-1), 5.29 (d, 1 H, *J* 3.9 Hz, H-1), 5.30 (d, 1 H, *J* 7.1 Hz, H-1a), 7.47 (d, 1 H, *J* 9.3 Hz, H-6 of CNP), 8.19 (dd, 1 H, *J* 9.3 Hz and 2.7 Hz, H-5 of CNP), 8.31 (d, 1 H, *J* 2.7 Hz, H-3 of CNP); ^{13}C NMR: δ 94.8 (C=*C*H_2); t_R (column *B*; eluent: 3:1

CH_3CN–H_2O): 9.8 min. Anal. Calcd for $C_{36}H_{52}ClNO_{27}$: C, 44.75; H, 5.42; N, 1.45. Found: C, 44.57; H, 5.66; N, 1.34.

O-(2,3-Di-O-acetyl-4,6-O-benzylidene-α-D-glucopyranosyl)-(1 → 4)-tris[O-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1 → 4)]-1,2,3,6-tetra-O-acetyl-D-glucopyranose (**5**).—Benzaldehyde dimethyl acetal (3.64 mL, 24.2 mmol) and *p*-toluenesulfonic acid monohydrate (750 mg, 3.95 mmol) were added to a stirred solution of maltopentaose (**4**; 10.0 g, 12.1 mmol) in DMF (150 mL), and the mixture was kept at 55°C for 4 h under reduced pressure (18 mmHg). Then, the reaction solution was slowly dropped into 2:1 EtOAc–CH_2Cl_2 (300 mL) containing Et_3N (2.50 mL, 17.8 mmol) with stirring to allow formation of the precipitate. The resulting suspension was filtered through glass-fiber filters to obtain an insoluble material, which was dissolved in pyridine (200 mL); acetic anhydride (100 mL, 1.06 mol) was then added and the mixture was kept at room temperature for 2 days. The solution was then evaporated in vacuo to leave a syrupy residue, which was chromatographed on SiO_2 gel with 15:85 MeOH–CH_2Cl_2 to give **5** (12.4 g, 8.02 mmol, 66.3% from **4**); mp 123–126°C (from Et_2O); $[\alpha]_D$ +125° (*c* 0.63, 1,4-dioxane); ν_{max} 2960 (CH, aliph.), 1752 (C=O), 1630 (arom.), 1430, 1374, 1238, and 1036 (C–O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.98–2.22 (cluster of s, 45 H, 15 OAc), 3.70–5.05 (m, 25 H, H-2a–e,4a–e,5a–e,6a–e), 5.22–5.60 (m, 10 H, H-1b–e,3a–e, and C*H*Ph), 5.75 (d, ~0.5 H, *J* 7.8 Hz, α H-1a), 6.24 (d, ~0.5 H, *J* 3.7 Hz, β H-1a), and 7.27–7.45 (m, 5 H, Ph); t_R (column *A*; detector: UV; eluent: 7:3 CH_3CN–H_2O): 6.2 min. Anal. Calcd for $C_{67}H_{86}O_{41}$: C, 52.01; H, 5.60. Found: C, 51.74; H, 5.58.

O-(2,3-Di-O-acetyl-4-O-benzoyl-6-bromo-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[O-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1 → 4)]-1,2,3,6-tetra-O-acetyl-D-glucopyranose (**6**).—Barium carbonate (14.8 g, 75.0 mmol) was added to a stirred solution of **5** (11.0 g, 7.50 mmol) in CCl_4 (600 mL) and 1,1,2,2-tetrachloroethane (300 mL), and the resulting mixture was heated under reflux with stirring. Then, *N*-bromosuccinimide (2.00 g, 11.2 mmol) was added to the refluxing solution and the reaction was carried out with refluxing for 2 h. The solution was cooled and filtered through a pad of Celite. The insoluble material was washed with CH_2Cl_2. The filtrate and washing were evaporated in vacuo to leave a syrupy residue, which was chromatographed on SiO_2 gel with 25:1:99 EtOAc–MeOH–CH_2Cl_2 to give **6** (9.88 g, 6.08 mmol, 81.1%); mp 117–119°C (from Et_2O), $[\alpha]_D$ +114° (*c* 0.64, 1,4-dioxane); ν_{max} 2960 (CH, aliph.), 1750 (C=O), 1372, 1238, and 1036 (C–O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.89–2.23 (cluster of s, 45 H, 15 OAc), 3.40–5.00 (m, 25 H, H-2a–e,4a–e,5a–e,6a–e), 5.20–5.65 (m, 9 H, H-1b–e,3a–e), 5.76 (d, ~0.5 Hz, *J* 8.1 Hz, α H-1a), 6.44 (d, ~0.5 H, *J* 3.4 Hz, β H-1a), 7.43 (dd, 2 H, *J* 7.8, 7.8 Hz, H-3 and H-5 of Bz), 7.56 (t, 1 H, *J* 7.8 Hz, H-4 of Bz), 8.00 (d, 2 H, *J* 7.8 Hz, H-2 and H-6 of Bz); t_R (column *A*; detector: UV; eluent: 7:3 CH_3CN–H_2O): 7.6 min. Anal. Calcd for $C_{67}H_{85}BrO_{41} \cdot 0.5H_2O$: C, 49.21; H, 5.30. Found: C, 49.15; H, 5.24.

O-(2,3-Di-O-acetyl-4-O-benzoyl-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[O-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1 → 4)]-1,2,3,6-tetra-O-acetyl-D-glucopyranose

(7).—A stirred solution of **6** (4.99 g, 3.07 mmol) in 1,4-dioxane (100 mL) was hydrogenolyzed immediately after the addition of 5% Pd/C (2.5 g) and $BaCO_3$ (2.5 g, 12.7 mmol) at atmospheric pressure and at 70°C for 3 days. The reaction solution was cooled and filtered through a pad of Celite. The insoluble material was washed with CH_2Cl_2. The filtrate and washings were combined and evaporated in vacuo to leave a syrupy residue, which was chromatographed on SiO_2 gel with 25:1:99 EtOAc–MeOH–CH_2Cl_2 to give **7** (4.22 g, 2.73 mmol, 88.9%); mp 110–114°C (from Et_2O); $[\alpha]_D$ +118° (*c* 0.65, 1,4-dioxane); ν_{max} 2960 (CH, aliph.), 1754 (C=O), 1372, 1238, and 1034 (C–O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.21 (3 H, d, *J* 5.9 Hz, H-6e), 1.87–2.34 (cluster of s, 45 H, 15 OAc), 3.80–5.05 (m, 23 H, H-2a–e,4a–e,5a–e,6a–d), 5.20–5.55 (9 H, m, H-1b–e,3a–e), 5.75 (d, ~0.5 H, *J* 8.1 Hz, α H-1a), 6.24 (d, ~0.5 H, *J* 3.9 Hz, β H-1a), 7.43 (dd, 2 H, *J* 7.6, 7.6 Hz, H-3 and H-5 of Bz), 7.56 (t, 1 H, *J* 7.6 Hz, H-4 of Bz), 8.00 (d, 2 H, *J* 7.6 Hz, H-2 and H-6 of Bz); t_R (column *A*; detector: UV eluent: 7:3 CH_3CN–H_2O): 7.1 min. Anal. Calcd for $C_{67}H_{86}O_4$: C, 52.01; H, 5.60. Found: C, 51.85; H, 5.59.

O-(2,3-Di-O-acetyl-4-O-benzoyl-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[O-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1 → 4)-2,3,6-tri-O-acetyl-α-D-glucopyranosyl bromide (**8**).—Phosphorus tribromide (PBr_3, 519 μL, 5.46 mmol) and H_2O (246 μL, 13.7 mmol) were added dropwise to a stirred solution of compound **7** (4.22 g, 2.73 mmol) in CH_2Cl_2 (150 mL), and the reaction was carried out at 30°C for 20 h. The mixture was cooled and neutralized with K_2CO_3, then filtered through a glass filter. The insoluble material was washed with CH_2Cl_2. The filtrate and washings were combined and evaporated in vacuo to leave a syrupy residue, which was chromatographed on SiO_2 gel with 25:1:99 EtOAc–MeOH–CH_2Cl_2 to give **8** (4.22 g, 2.73 mmol, 88.9%); mp 108–110°C (from Et_2O); $[\alpha]_D$ +155° (*c* 0.96, 1,4-dioxane); ν_{max} 2960 (CH, aliph.), 1752 (C=O), 1370, 1236, and 1034 (C–O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 (d, 3 H, *J* 6.4 Hz, H-6e), 1.73–2.31 (cluster of s, 42 H, 14 OAc), 3.85–5.20 (m, 23 H, H-2a–e,4a–e,5a–e,6a–d), 5.25–5.65 (m, 9 H, H-1b–e,3a–e), 6.50 (d, 1 H, *J* 4.2 Hz, H-1a), 7.42 (dd, 2 H, *J* 7.5, 7.5 Hz, H-3 and H-5 of Bz), 7.55 (t, 1 H, *J* 7.5 Hz, H-4 of Bz), 8.00 (d, 2 H, *J* 7.5 Hz, H-2 and H-6 of Bz); t_R (column *A*; detector: UV; eluent: 7:3 CH_3CN–H_2O): 9.0 min. Compound **8** was unstable so that it could not be characterized by elemental analysis.

2-Chloro-4-nitrophenyl O-(2,3-di-O-acetyl-4-O-benzoyl-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[O-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1 → 4)]-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**9**).—2-Chloro-4-nitrophenol (CNP, 2.25 g, 12.9 mmol) and Ag_2O (3.00 g, 12.9 mmol) were added to a stirred solution of **8** (3.92 g, 2.50 mmol) in CH_3CN (150 mL), and the reaction was carried out with stirring at 40°C for 15 h. The mixture was cooled and filtered through a pad of Celite. The insoluble material was washed with CH_2Cl_2. The filtrate and washings were combined and evaporated in vacuo to leave a syrupy residue, which was chromatographed on SiO_2 gel with 25:1:99 EtOAc–MeOH–CH_2Cl_2 to give **9** (3.40 g, 2.05 mmol, 82.0%); mp 118–121°C (from Et_2O); $[\alpha]_D$ +84.4° (*c* 0.63, 1,4-dioxane); ν_{max} 2960 (CH, aliph.), 1756 (C=O), 1528 and 1350 (NO_2), 1370, 1234, and 1034

(C–O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.17 (d, 3 H, *J* 6.1 Hz, H-6e), 1.83–2.20 (cluster of s, 42 H, 14 OAc), 3.90–5.20 (m, 23 H, H-2a–e,4a–e,5a–e,6a–d), 5.25–5.60 (m, 10 H, H-1a–e,3a–e), 7.29 (d, 1 H, *J* 9.0 Hz, H-6 of CNP), 7.44 (dd, 2 H, *J* 7.5, 7.5 Hz, H-3 and H-5 of Bz), 7.57 (t, 2 H, *J* 7.5 Hz, H-4 of Bz), 7.99 (d, 2 H, *J* 7.5 Hz, H-2 and H-6 of Bz), 8.15 (dd, 1 H, *J* 9.0, 2.9 Hz, H-5 of CNP), 8.29 (d, 1 H, *J* 2.9 Hz, H-3 of CNP); t_R (column *A*; detector: UV; eluent: 7:3 CH_3CN–H_2O): 15.5 min. Anal. Calcd for $C_{71}H_{86}ClNO_{42}$: C, 51.34; H, 5.22; N, 0.84. Found: C, 51.06; H, 5.19; N, 0.58.

2-Chloro-4-nitrophenyl O-*(6-deoxy-α-*D-*glucopyranosyl)-(1 → 4)-tris[*O-*α-*D-*glucopyranosyl-(1 → 4)]-β-*D-*glucopyranoside* (**10**).–Aqueous ammonia (28%, 25.0 mL) and H_2O (13.0 mL) were added to a stirred solution of **9** (3.40 g, 2.05 mmol) in MeOH (50 mL), and the reaction was carried out with stirring at 35°C for 20 h. The reaction solution was evaporated in vacuo to leave a syrupy residue, which was chromatographed on ODS gel with 1:4 EtOH–H_2O to give **10** (1.43 g, 1.48 mmol, 72.2%) as colorless prisms; mp 188–191°C (from H_2O); $[\alpha]_D$ +95.0° (*c* 0.37, H_2O); ν_{max} 3430 (OH), 2940 (CH, aliph.), 1644, 1588, and 1488 (arom.), 1522 and 1352 (NO_2), 1276, 1252, 1154, 1082, and 1046 (C–O) cm^{-1}; ^1H NMR (D_2O): δ 1.28 (d, 3 H, *J* 5.9 Hz, H-6e), 3.14–4.00 (m, 28 H, H-2a–e,3a–e,4a–e,5a–e,6a–d), 5.28–5.35 (m, 4 H, H-1b–e), 5.43 (d, 1 H, *J* 6.6 Hz, H-1a), 7.40 (d, 1 H, *J* 9.2 Hz, H-6 of CNP), 8.22 (dd, 1 H, *J* 9.2 Hz, 2.7 Hz, H-5 of CNP), 8.40 (d, 1 H, *J* 2.7 Hz, H-3 of CNP); t_R (column *B*; detector: UV; eluent: 3:1 CH_3CN–H_2O): 9.2 min. Anal. Calcd for $C_{36}H_{54}ClNO_{27}$: C, 44.66; H, 5.62; N, 1.45. Found: C, 44.59; H, 5.70; N, 1.44.

*6-*O*-*p*-Toluenesulfonylcyclomaltoheptaose* (**12**).—*p*-Toluenesulfonyl chloride (10.0 g, 52.4 mmol) was added to a stirred solution of cyclomaltoheptaose (**11**; 5.00 g, 4.41 mmol) in pyridine (50 mL), and the mixture was continuously stirred at room temperature for 5 h. Then, the reaction solution was evaporated in vacuo to leave a syrupy residue, to which H_2O (100 mL) and benzene (150 mL) were added with stirring to precipitate a solid. The solid was collected by filtration through a glass filter, washed with acetone (100 mL), and then chromatographed on ODS gel with 1:9 EtOH–H_2O to give **12** (1.63 g, 1.26 mmol, 28.6%); mp 172–174°C (dec) (from H_2O); ν_{max} 3400 (OH), 2930 (CH, aliph.), 1642, 1632, 1600, and 1424 (arom.), 1360 and 1178 (S=O), 1156, 1078, 1028 (C–O) cm^{-1}; ^1H NMR: δ 2.44 (s, 3 H, CH_3 of Ts), 3.15–4.45 (m, ~40 H, H-2–6), 4.76 and 4.85 (br s, 1 H and 6 H, H-1), 7.44 (d, 2 H, *J* 8.8 Hz, H-3 and H-5 of Ts), 7.75 (d, 2 H, *J* 8.8 Hz, H-2 and H-6 of Ts). Anal. Calcd for $C_{49}H_{76}O_{37}S$: C, 45.65; H, 5.94. Found: C, 45.95; H, 5.84.

6-Deoxycyclomaltoheptaose (**13**).—Sodium borohydride ($NaBH_4$, 384 mg, 10.2 mmol) was added to a stirred solution of **12** (1.27 g, 0.985 mmol) in Me_2SO (20 mL), and the mixture was further stirred at 50°C for 12 h. Then, H_2O (1.0 L) was added to the solution and the resulting mixture was subjected to ODS gel chromatography in order to remove the Me_2SO, followed by purification. The appropriate fractions eluted with 1:9 EtOH–H_2O were concentrated to give **13** (840 mg, 0.750 mmol, 76.1%); mp 280–281°C (dec) (from H_2O); ν_{max} 3370 (OH),

2920 (CH, aliph.), 1152, 1080, and 1020 (C–O) cm^{-1}; ^1H NMR: δ 1.20 (d, 3 H, J 5.9 Hz, H-6e), 2.80–4.05 (m, ca. 40 H, H-2–6), 4.84 (br s, 7 H, H-1). Anal. Calcd for $C_{42}H_{70}O_{34}$: C, 45.08; H, 6.31. Found: C, 44.99; H, 6.45.

6-Deoxy-D-glucopyranose (**14**), *O-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-D-glucopyranose* (**15**), *O-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-O-(α-D-glucopyranosyl)-(1 → 4)-D-glucopyranose* (**16**), *O-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-bis[O-α-D-glucopyranosyl-(1 → 4)]-D-glucopyranose* (**17**), *O-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris[O-α-D-glucopyranosyl-(1 → 4)]-D-glucopyranose* (**18**), *O-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tetrakis[O-α-D-glucopyranosyl-(1 → 4)]-D-glucopyranose* (**19**), *and O-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-pentakis[O-α-D-glucopyranosyl-(1 → 4)]-D-glucopyranose* (**20**).—CDase[14] (~ 100 U) was added to a stirred solution of **13** (63.4 g, 56.8 mmol) in 100 mM phosphate buffer (pH 7.0, 1.0 L), and the mixture was stirred at 40°C for 48 h. The solution was adjusted to pH 2.0 with 0.5 M HCl to stop the reaction, neutralized with 0.5 M NaOH, passed through an ODS gel column to absorb the unreacted **13**, and then mixed with one-tenth of its volume of 100 mM acetate buffer (pH 4.5). The pH of the resulting mixture was adjusted to 4.5 with AcOH. Then, glucoamylase (2500 U) was added and the mixture was subjected to enzymatic reaction at 40°C for 8 h. The solution was adjusted to pH 2.0 with 0.5 M HCl to stop the reaction, neutralized with 0.5 M NaOH, and then passed through a column of active carbon. 6-Deoxy derivatives were eluted using an EtOH gradient of 0 to 35%, and the appropriate fractions were freeze-dried to give **14** (1.34 g, 8.27 mmol, 14.6%), **15** (2.64 g, 8.10 mmol, 14.2%), **16** (1.10 g, 2.25 mmol, 4.0%), **17** (2.43 g, 3.74 mmol, 6.6%), **18** (2.45 g, 3.02 mmol, 5.3%), **19** (2.28 g, 2.34 mmol, 4.1%), **20** (2.68 g, 2.36 mmol, 4.2%), and D-glucopyranose.

Compound **14** was colorless and amorphous, and had: $[\alpha]_D$ +31.8° (*c* 0.50, H_2O); ν_{max} 3370 (OH), 3120, 2960 (CH, aliph.), 1452, 1368, 1264, 1170, 1148, 1114, 1040, and 1008 (C–O) cm^{-1}; ^1H NMR (D_2O at 80°C): δ 1.25 and 1.29 (d, each ~ 1.5 H, J 6.3 Hz, H-6), 3.10–3.92 (m, 4 H, H-2–5), 4.62 (d, ~ 0.5 Hz, J 6.7 Hz, α H-1), 5.17 (d, ca. 0.5 H, J 3.7 Hz, β H-1); t_R (column *B*: detector: ri; eluent: 3:2 CH_3CN–H_2O): 4.2 min. Anal. Calcd for $C_6H_{12}O_5 \cdot 0.14H_2O$: C, 43.22; H, 7.43. Found: C, 43.21; H, 7.30.

Compound **15** was colorless and amorphous, and had: $[\alpha]_D$ +132° (*c* 0.50, H_2O); ν_{max} 3420 (OH), 2930 (CH, aliph.), 1364, 1146, and 1046 (C–O) cm^{-1}; ^1H NMR (D_2O at 80°C): δ 1.28 (d, 3 H, J 6.4 Hz, H-6b), 3.12–3.95 (m, 10 H, H-2a–b to H-5a–b and H-6a), 4.65 (d, ~ 0.5 Hz, J 7.2 Hz, α H-1), 5.23 (d, ~ 0.5 H, J 3.7 Hz, β H-1a), 5.27 (d, 1 H, J 3.2 Hz, H-1b); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 5.0 min. Anal. Calc. for $C_{12}H_{22}O_{10} \cdot 0.3H_2O$: C, 43.37; H, 6.88. Found: C, 43.33; H, 6.89.

Compound **16** was colorless and amorphous and had: $[\alpha]_D$ +158° (*c* 0.51, H_2O); ν_{max} 3400 (OH), 2950 (CH, aliph.), 1362, 1146, and 1042 (C–O) cm^{-1}; ^1H NMR (D_2O at 80°C): δ 1.28 (d, 3 H, J 6.1 Hz, H-6c), 3.12–4.00 (m, 16 H, H-2a–c to H-5a–c and H-6a–b), 4.65 (d, ~ 0.5 H, J 7.8 Hz, α H-1a), 5.24 (d, ~ 0.5 Hz, J 3.7

Hz, β H-1a), 5.27 (d, 1 H, *J* 2.9 Hz, H-1b), 5.36 (d, 1 H, *J* 3.9 Hz, H-1c); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 5.9 min. Anal. Calcd for $C_{18}H_{32}O_{15} \cdot H_2O$: C, 42.69; H, 6.77. Found: C, 42.79; H, 6.70.

Compound **17** was colorless and amorphous, and had: $[\alpha]_D$ +169° (*c* 0.50, H_2O); ν_{max} 3420 (OH), 2930 (CH, aliph.), 1410, 1366, 1148, and 1038 (C–O) cm^{-1}; ^1H NMR (D_2O at 80°C): δ 1.27 (d, 3 H, *J* 6.1 Hz, H-6d), 3.13–4.05 (m, 22 H, H-2a–d to H-5a–d and H-6a–c), 4.63 (d, ~0.5 H, *J* 7.6 Hz, α H-1a), 5.22 (d, ~0.5 H, *J* 3.5 Hz, β H-1a), 5.25 (d, 1 H, *J* 2.9 Hz, H-1b), 5.36 (br s, 2 H, H-1c and 1d); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 7.2 min. Anal. Calcd for $C_{24}H_{42}O_{20} \cdot H_2O$: C, 43.12; H, 6.63. Found: C, 43.21; H, 6.52.

Compound **18** was colorless and amorphous, and had: $[\alpha]_D$ +175° (*c* 0.51, H_2O); ν_{max} 3420 (OH), 2925 (CH, aliph.), 1412, 1368, 1150, and 1040 (C–O) cm^{-1}; ^1H NMR (D_2O at 80°C): δ 1.27 (d, 3 H, *J* 6.3 Hz, H-6e), 3.16–4.10 (m, 28 H, H-2a–e to H-5a–e and H-6a–d), 4.65 (d, ~0.5 H, *J* 8.1 Hz, α H-1a), 5.23 (d, ~0.5 H, *J* 3.4 Hz, β H-1a), 5.27 (d, 1 H, *J* 3.2 Hz, H-1b), 5.35 (d, 3 H, *J* 3.9 Hz, H-1c–e); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 8.7 min. Anal. Calcd for $C_{30}H_{52}O_{25} \cdot H_2O$: C, 43.38; H, 6.55. Found: C, 43.26; H, 6.53.

Compound **19** was colorless and amorphous, and had: $[\alpha]_D$ +181° (*c* 0.50, H_2O); ν_{max} 3400 (OH), 2930 (CH, aliph.), 1410, 1362, 1150, and 1036 (C–O) cm^{-1}; ^1H NMR (D_2O at 80°C): δ 1.28 (d, 3 H, *J* 6.1 Hz, H-6f), 3.12–4.10 (m, 34 H, H-2a–f to H-5a–f and H-6a–e), 4.65 (d, ~0.5 H, *J* 8.3 Hz, α H-1a), 5.22 (d, >0.5 H, *J* 3.5 Hz, β H-1a), 5.27 (d, 1 H, *J* 2.9 Hz, H-1b), 5.35 (4 H, d, *J* 3.7 Hz, H-1c–f); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 10.1 min. Anal. Calcd for $C_{36}H_{62}O_{30} \cdot 1.5H_2O$: C, 43.16; H, 6.54. Found: C, 43.16; H, 6.51.

Compound **20** was colorless and amorphous, and had: $[\alpha]_D$ +183° (*c* 0.50, H_2O); ν_{max} 3420 (OH), 2930 (CH, aliph.), 1412, 1362, 1150, 1078, and 1022 (C–O) cm^{-1}; ^1H NMR (D_2O at 80°C): δ 1.28 (d, 3 H, *J* 6.2 Hz, H-6g), 3.13–4.10 (m, 40 H, H-2a–g to H-5a–g and H-6a–f), 4.65 (d, ~0.5 H, *J* 8.1 Hz, α H-1a), 5.23 (d, ~0.5 H, *J* 3.4 Hz, β H-1a), 5.28 (d, 1 H, *J* 2.7 Hz, H-1b), 5.35 (5 H, d, *J* 3.8 Hz, H-1c–g); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 12.1 min. Anal. Calcd for $C_{42}H_{72}O_{35} \cdot 1.5H_2O$: C, 43.34; H, 6.49. Found: C, 43.11; H, 6.44.

O-(2,3,4-Tri-O-acetyl-6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris-[O-(2,3,6-tri-O-acetyl-α-D-glucopyranosyl)-(1 → 4)]-1,2,3,6-tetra-O-acetyl-D-glucopyranose (**21**).—Acetic anhydride (15.0 mL) was added to a stirred solution of **18** (821 mg, 1.01 mmol) in pyridine (30 mL), and the mixture was kept at room temperature for 2 days. Then, the solution was evaporated in vacuo to leave a syrupy residue, which was chromatographed on SiO_2 gel with 1.5:100 MeOH–CH_2Cl_2 to give **21** (1.37 g, 0.923 mmol, 92.3%); mp 111–115°C (from Et_2O); $[\alpha]_D$ +131° (*c* 0.51, 1,4-dioxane); ν_{max} 2970 (CH, aliph.), 1754 (C=O), 1372, 1236, and 1038 (C–O) cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.15 (d, 3 H, *J* 6.1 Hz, H-6e), 1.75–2.25 (cluster of s, 48 H, 16 OAc), 3.70–5.10 (m, 23 H, H-2a–e,4a–e,5a–e,6a–d), 5.22–5.58 (m, 9 H, H-1b–e and 3a–e), 5.76 (d, ~0.5 H, *J* 8.1 Hz, α H-1a), 6.24 (d, ~0.5 H, *J* 3.5 Hz, β H-1a); t_R

(column *A*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 9.4 min. Anal. Calcd for $C_{62}H_{84}O_{41}$: C, 50.14; H, 5.70. Found: C, 49.90; H, 5.68.

2-Chloro-4-nitrophenyl O-(6-deoxy-α-D-glucopyranosyl)-(1 → 4)-tris-[O-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**10**).—Reaction of **21** (1.32 g, 0.899 mmol) with PBr_3 was done as described for **8**, to give the glycosyl bromide, which was treated with 2-chloro-4-nitrophenol and Ag_2O as described for **9**; subsequent *O*-deacetylation with ammonia–H_2O, as described above, afforded **10** (447 mg, 0.463 mmol, 51.5% from **21**); mp 189–192°C (from H_2O). The other physical properties and spectral data were all in agreement with those of **10** obtained via **9**, as described above.

3,6-Anhydrocyclomaltoheptaose (**22**).—To a stirred solution of **12** (21.0 g, 16.3 mmol) in 3:1 H_2O—MeOH (1.0 L) was added M KOH (350 mL), and the mixture was stirred at 50°C for 4 h. The basic solution was neutralized with M HCl, and half of the solvent was evaporated in vacuo in order to remove MeOH. The remaining solution was chromatographed on an ODS gel column (H_2O → 1.5% → 3% → 4% → 4.5% CH_3CN, stepwise) to give **22** (14.9 g, 13.4 mmol, 82.2%); $[\alpha]_D$ +139° (*c* 0.544, H_2O); ν_{max} 3410 (OH), 2920 (CH, aliph.), 1154, 1078, and 1028 (C–O) cm^{-1}; ^1H NMR: δ 3.20–4.45 (m, ~40 H, H-2–6), 4.83 (br d, 4 H, *J* 3.4 Hz, H-1), 4.91 (d, 1 H, *J* 3.9 Hz, H-1), 4.94 (d, 1 H, *J* 4.4 Hz, H-1), 5.02 (br s, 1 H, H-1). Anal. Calcd for $C_{42}H_{68}O_{34} \cdot 2H_2O$: C, 43.75; H, 6.29. Found: C, 43.90; H, 6.28.

O-(3,6-anhydro-α-D-glucopyranosyl)-(1 → 4)-bis[O-α-D-glucopyranosyl-(1 → 4)]-D-glucopyranose (**23**), *O-(3,6-anhydro-α-D-glucopyranosyl)-(1 → 4)-tris[O-α-D-glucopyranosyl-(1 → 4)]-D-glucopyranose* (**24**), and *O-(3,6-anhydro-α-D-glucopyranosyl)-(1 → 4)-tetrakis[O-α-D-glucopyranosyl-(1 → 4)]-D-glucopyranose* (**25**).—CDase[14] (~600 U) was added to a stirred solution of **22** (15.0 g, 13.4 mmol) in 100 mM phosphate buffer (pH 7.8, 1.5 L), and the mixture was stirred at 40°C for 4 h. The solution was heated to 80°C to stop the reaction, passed through an ODS gel column to absorb the unreacted **22**, and then mixed with one-tenth of its volume of 100 mM acetate buffer (pH 4.5). The pH of the resulting mixture was adjusted to 4.5 with AcOH. Then, hydrolysis of the mixture with glucoamylase (2000 U) and subsequent column chromatography, as described for **14–20**, gave **23** (940 mg, 1.45 mmol, 10.8%), **24** (3.67 g, 4.53 mmol, 33.8%), **25** (2.59 g, 2.66 mmol, 19.9%), and D-glucopyranose.

Compound **23** was colorless and amorphous, and had: $[\alpha]_D$ +131° (*c* 0.51, H_2O); ν_{max} 3420 (OH), 2930 (CH, aliph.), 1366, 1150, 1078, and 1026 (C–O) cm^{-1}; ^1H NMR (D_2O): δ 3.24–4.05 (m, 24 H, H-2a–d to H-6a–d), 4.65 (d, ~0.5 H, *J* 7.9 Hz, α H-1a), 5.14 (d, 1 H, *J* 3.6 Hz, H-1d), 5.23 (d, ~0.5 H, *J* 3.6 Hz, β H-1a), 5.37 (d, 1 H, *J* 3.4 Hz, H-1), 5.38 (d, 1 H, *J* 2.4 Hz, H-1); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 7.5 min. Anal. Calcd for $C_{24}H_{40}O_{20} \cdot H_2O$: C, 43.25; H, 6.35. Found: C, 42.97; H, 6.31.

Compound **24** was colorless and amorphous, and had: $[\alpha]_D$ +146° (*c* 0.52, H_2O); ν_{max} 3400 (OH), 2930 (CH, aliph.), 1416, 1366, 1252, 1154, 1080, and 1024

(C–O) cm^{-1}; ^1H NMR (D_2O): δ 3.21–4.00 (m, 30 H, H-2a–e to H-6a–e), 4.64 (d, ~0.5 H, *J* 7.9 Hz, α H-1a), 5.14 (d, 1 H, *J* 3.6 Hz, H-1e), 5.23 (d, ~0.5 H, *J* 3.6 Hz, β H-1a), 5.35 (d, 2 H, *J* 5.1 Hz, H-1), 5.38 (d, 1 H, *J* 3.6 Hz, H-1); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 9.1 min. Anal. Calcd for $C_{30}H_{50}O_{25} \cdot 0.6H_2O$: C, 43.80; H, 6.29. Found: C, 43.80; H, 6.37.

Compound **25** was colorless and amorphous, and had: $[\alpha]_D$ +158° (*c* 0.51, H_2O); ν_{max} 3410 (OH), 2930 (CH, aliph.), 1410, 1368, 1250, 1152, 1080, and 1024 (C–O) cm^{-1}; ^1H NMR (D_2O): δ 3.22–4.10 (m, 36 H, H-2a–f to H-6a–f), 4.65 (d, ~0.5 H, *J* 7.9 Hz, α H-1a), 5.14 (d, 1 H, *J* 3.6 Hz, H-1f), 5.23 (d, ~0.5 H, *J* 3.8 Hz, β H-1a), 5.36 (d, 3 H, *J* 3.8 Hz, H-1), 5.38 (d, 1 H, *J* 3.4 Hz, H-1); t_R (column *B*; detector: ri; eluent: 3:2 CH_3CN–H_2O): 11.1 min. Anal. Calcd for $C_{36}H_{60}O_{30} \cdot 1.5H_2O$: C, 43.25; H, 6.35. Found; C, 43.33; H, 6.33.

2-Chloro-4-nitrophenyl O-(3,6-anhydro-α-D-glucopyranosyl)-(1 → 4)-tris[O-α-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranoside (**26**).—*O*-Acetylation of **24** (2.30 g, 2.84 mmol) with acetic anhydride in pyridine as described for **21**, followed by bromination with PBr_3, as described for **8**, gave $3^5,6^5$-anhydro-*O*-acetyl-maltopentaosyl bromide, which was glycosylated with 2-chloro-4-nitrophenol and Ag_2O as described for **9**. Subsequent *O*-deacetylation with ammonia–H_2O as described for **10**, afforded **26** (1.17 g, 1.16 mmol, 40.9% from **24**); pale-yellow, amorphous; $[\alpha]_D$ +71.2° (*c* 0.504, H_2O); ν_{max} 3410 (OH), 2930 (CH, aliph.), 1640, 1586, and 1486 (arom.), 1522 and 1352 (NO_2), 1276, 1252, 1154, 1076, and 1022 (C–O) cm^{-1}; ^1H NMR: δ 3.05–4.15 (m, 30 H, H-2a–e to H-6a–e), 4.93 (d, 1 H, *J* 3.9 Hz, H-1), 5.06 (d, 1 H, *J* 3.7 Hz, H-1), 5.12 (d, 1 H, *J* 4.2 Hz, H-1), 5.25 (br s, 1 H, H-1), 5.27 (d, 1 H, *J* 7.8 Hz, H-1a), 7.47 (d, 1 H, *J* 9.3 Hz, H-6 of CNP), 8.19 (dd, 1 H, *J* 9.3 and 2.6 Hz, H-5 of CNP), 8.31 (d, 1 H, *J* 2.6 Hz, H-3 of CNP); ^{13}C NMR: δ 68.4 ($CHCH_2OC$); t_R (column *B*; detector: UV; eluent: 3:1 CH_3CN–H_2O): 9.7 min. Anal. Calcd for $C_{36}H_{52}ClNO_{27} \cdot 1.5H_2O$: C, 43.53; H, 5.58; N, 1.41. Found: C, 43.32; H, 5.42; N, 1.37.

Michaelis constants (K_m).—A solution of coupled enzymes (110 U/mL α-D-glucosidase and 13 U/mL β-D-glucosidase, 1.0 mL) in 50 mM phosphate buffer (pH 7.0, containing 40 mM NaCl and 2.0 mM $MgCl_2$) was added to a solution of HPA or HSA in H_2O (150 U/mL, 0.25 mL), and the enzymic solution was incubated at 37°C for 1 min. Then, a solution of **3**, **10**, or **26** (2.0 mL) in the same buffer was added to the enzymic solution and the mixture was incubated. After 2 min, the reaction was monitored by the increase in absorbance at 400 nm for 2 min. For the blank, H_2O was added instead of the amylase solution. The K_m values of hydrolysis of the substrates were calculated by the method of least squares, using Lineweaver–Burk plots as shown in Table I.

Patterns of action.—A solution of **3** or **10** (1.5 mM each, 2.0 mL) in the same buffer was added to a solution of HPA or HSA in H_2O (150 U/mL, 0.25 mL), and the mixture was incubated at 37°C for 15 min. A portion (0.1 mL) of the mixture was then added to CH_3CN (0.9 mL) in order to stop the action. The sample (5 μL) was analyzed by HPLC; the patterns of action of the substrates are summarized in Table II.

Relative rate of hydrolysis.—A solution of the coupled enzymes (1.0 mL) described above was added to a solution of HPA or HSA in H_2O (0.25 mL), and the enzymic solution was incubated at 37°C for 1 min. Then, a solution (2.0 mL) of **3** (1.5 mM), **10** (0.40 mM), or **27** (2.0 mM) in the buffer described above was added to the enzymic solution, and the mixture was incubated. After 2 min, the reaction was monitored by the increase in absorbance at 400 nm for 2 min. For the blank, H_2O was added instead of the amylase solution. Relative rates of hydrolysis are shown in Table I.

Differential assay of HPA and HSA, using eqs 3 *and* 4.—A solution of the coupled enzymes described above was added to a sample solution (0.25 mL) containing known amounts of HPA and/or HSA in H_2O (total activity, 130 U/L; activity of HPA/HSA, 0/10–10/0, 11 samples), or a sample from human serum (50 persons), and the enzymic solution was incubated at 37°C for 1 min. Then, using a substrate solution (2.0 mL) of **10** (0.40 mM) or **27** (2.0 mM), the increase in absorbance at 400 nm was determined by the above method. From the values for A_1 and A_2, values of a_p and a_s were calculated using eqs *3* and *4*, and k. These results are summarized in Figs. 2, 3, and 4.

Differential assay of HPA by the method (enzyme immunoassay method) using monoclonal antibodies of HSA.—The assay was carried out following the directions for use of the enzyme immunoassay-kit. These results are summarized in Fig. 4.

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