Isopropylidene Maltoheptosyl Fructofuranoside, Doubly Blocked Substrate for Determination of Endoamylase Activity

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We synthesized \(\alpha\)-(6,8-\alpha\)-isopropylidene-\alpha\-D-glucopyranosyl)-(1\rightarrow4)-(\alpha\-D-glucosyl-(1\rightarrow4)]_{\text{G5F}}, \alpha\-D-glucopyranosyl-(1\rightarrow2)-\alpha\-D-fructofuranoside (IPG7F) and developed an assay for determining the activity of amylase in human serum and urine by using this substrate. Glucoamylase, \alpha\-glucosidase, and mannotelid dehydrogenase are used as coupling enzymes. The coupled reactions are monitored by continuously measuring the oxidation rate of NADH. In this procedure, various substances in the test specimens do not interfere with the detection of amylase activity. Exactly one molecule of NADH is oxidized by one attack of amylase on the substrate, although four products can be produced in the reaction. The within-assay coefficient of variation (CV) ranged from 1.0% to 4.1% and the between-assay CV ranged from 2.6% to 5.3%. The results of our new assay correlate well with those of the amylase assay involving \(\alpha\)-nitrophenol maltoheptide as substrate (\(r = 0.978\)) and with those of the amylase assay involving maltopentose (\(r = 0.987\)).

Additional Keyphrases: enzyme activity · synthetic substrate

\(\alpha\)-Nitrophenyl maltoheptose (G5pNP) and \(\alpha\)-nitrophenyl maltotriose (G7pNP) are widely used as substrates for determination of amylase (AMY; 1,4-\alpha\-D-glucan glucanohydrolase, EC 3.2.1.1) activity in human serum and urine.4 However, the methods based on use of these substrates have several problems. First, the stability of the reagents after mixing is very poor because the substrates, which are not blocked at their nonreducing ends, are hydrolyzed by \(\alpha\)-glucosidase (EC 3.2.1.20). To solve this problem, the nonreducing end of the substrate can be blocked so that it cannot be hydrolyzed by \(\alpha\)-glucosidase. In fact, various substrates blocked at their nonreducing ends have been synthesized (1-4) since our report on this approach (5). However, when a substrate is blocked at its nonreducing end with benzylidene, the rate of its hydrolysis by AMY is slowed. We have now succeeded in synthesizing the more chemically stable \(\alpha\)-(6,8-\alpha\)-isopropylidene-\alpha\-D-glucopyranosyl)-(1\rightarrow4)-(\alpha\-D-glucopyranosyl)-(1\rightarrow4)/(\alpha\-D-glucopyranosyl)-(1\rightarrow2)-\alpha\-D-fructofuranoside (IPG7F). This compound is a maltosehexose blocked at its nonreducing end with 4,6-\alpha\-isopropylidene-\alpha\-D-glucose group and at its reducing end with a fructose moiety; the rate of its hydrolysis is threefold greater than that of benzylidated \(\alpha\)-nitrophenyl maltotriose.

A second problem with the use of G5pNP and G7pNP is the difficulty of determining \(\alpha\)-nitrophenol (pNP) accurately. Because the \(pK_a\) value of pNP is in the range of about pH 7.0, the molar absorptivity of pNP varies with even a slight change of pH, resulting in a change in the apparent AMY activity. Similarly, because pH values are temperature dependent, a slight change in temperature also indirectly affects the determination of AMY activity (6). Other factors also affect pNP absorbance: ionic strength, various proteins (7), and surfactants (7). In the most remarkable case, the absorbance doubles with the addition of \(\alpha\)-cyclodextrin (7). Furthermore, because the estimation of pNP is affected by the presence of hemoglobin (8), pNP cannot be measured accurately in hemolyzed blood specimens or in urine specimens containing blood.

To solve these problems, we have developed a continuous monitoring method based on oxidation of NADH. IPG7F is hydrolyzed by AMY to produce four products: maltotetrasyl fructofuranoside (G5F), maltotetraosyl fructofuranoside (G4F), maltotriosyl fructofuranoside (G3F), and maltosyl fructofuranoside (G2F), collectively referred to as G4,F. These products are completely hydrolyzed by \(\alpha\)-glucosidase and glucoamylase to release fructose. Fructose is then reacted with NADH in the presence of mannitol dehydrogenase (EC 1.1.1.67) to form mannitol and NAD\(^+\). Thus AMY activity is assayed by monitoring the oxidation rate of NADH.

Materials and Methods
Reagents and Instruments

Human pancreatic AMY was purified by the method of Stiefel and Keller (9). Collected human parotid saliva was used for the purification of salivary AMY according to the method of Fischer and Stein (10). Specific activi...
ties of the two isoenzymes were 640 kU/g for pancreatic AMY and 580 kU/g for salivary AMY. AMY activity was assayed by the maltpentaose (G5)→hexokinase method (11), and the concentration of protein was determined by the method of Lowry et al. (12). AMY was diluted with 1.4-piperazineethanesulfonic acid (PIPS) buffer (10 mmol/L, pH 7.0) containing, per liter, 1.0 mmol of NaCl, 0.1 mmol of CaCl2, and 1.0 g of human albumin. Glucoamylase from *Rhizopus delemar* and α-glucosidase from *Saccharomyces carlsbergensis* were purchased from Toyobo Co. (Osaka, Japan). NADH was provided by Oriental Yeast Co. (Tokyo, Japan). The other reagents were from Wako Pure Chemical Industries (Osaka, Japan). These reagents were all of analytical grade. Q-PAK control serum (Hyland Diagnostics, Deerfield, CA) and Monitrol (Dade Diagnostics, Miami, FL) were used as the control sera for the precision assay.

For the correlation studies, we used the G7pNP method (kit from Boehringer Mannheim GmbH, Mannheim, F.R.G.) and G5→hexokinase method (kit from Sanko Junyaku Co., Tokyo, Japan), measuring the activity in a Model UV-220A spectrophotometer equipped with a cell programmer (Hitachi Ltd., Tokyo, Japan) or Cobas-Bio centrifugal automated analyzer (F. Hoffmann-La Roche & Co., Ltd., Basel, Switzerland). The structures of IPG7F and maltolheptaosyl fructofuranoside (G7F) were evaluated with a Model JNM-GX 270 type ¹H, ¹³C nuclear magnetic resonance (NMR) spectroscopy (JEOL, Tokyo, Japan).

**Synthesis of IPG7F**

G7F was prepared as follows: to 4.0 L of 5 mmol/L phosphate buffer (pH 7.0) we added 200 g each of β-cyclodextrin and sucrose, and 5000 U of cyclomalto-dextrin glucanotransferase (CGTase; EC 2.4.1.19, from *Bacillus ohbensis*; purchased from Sanraku Inc., Tokyo, Japan). After stirring the mixture for 22 h at 60 °C, we increased the temperature to 100 °C for 3 min to deactivate the enzyme. Then, after filtration, we subjected the product to HPLC for 180 min, using a Model NSP-800 DX pump (Nihon Seimitsu Kagaku Ltd., Tokyo, Japan); a Model ERC-7520 refractive index detector (Erma Optical Co. Ltd., Tokyo, Japan); a YMC S-343 53 mm × 1.0 m octadecyl silane (ODS) column (particle diameter, 50 μm; Yamamura Chemical Co., Kyoto, Japan) and eluting with distilled water at a flow rate of 48 mL/min.

We formed the isopropylidene derivative of G7F as described for the derivatization of sucrose (13, 14). In this procedure, we dissolved 5.0 g of G7F in 50 mL of distilled dimethylformamide, added 36 mg of p-toluene-sulfonic acid and 1.1 mL of isopropenyl methyl ether (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan), and stirred the reaction mixture for 2 h at room temperature before adding 1.0 mL of triethylamine to stop the reaction. To check the progress of the reaction, we used thin-layer chromatography (TLC) on silica gel, developing with a solution of ethyl acetate and 700 mL/L isopropanol (2/1 by vol). The solvent was removed by azeotropic distillation with toluene in a rotary evaporator. The resulting white powder (IPG7F) was redissolved in distilled water and injected into the HPLC, being eluted after 37 min. For this chromatography we used a Model LC-6A pump (Shimadzu Seisakusyo Co. Ltd., Kyoto, Japan), the same detector as before, and a 20 × 250 mm YMC S-343 ODS column (Yamamura Chemical Co.). Elution was achieved with methanol, 120 mL/L, at a flow rate of 9.9 mL/min.

**Determination of AMY Activity**

AMY activity was assessed by measuring the oxidation rate of NADH at 340 nm. The reagent mixture (pH 7.0) contained 4.4 μmol of IPG7F, 11 U of mannitol dehydrogenase, 2.8 U of glucoamylase, 179 U of α-glucosidase, 0.178 μmol of NADH, 22 μmol of NaCl, 1.1 μmol of CaCl2, and 10 μmol of PIPES in a volume of 0.9 mL. The reaction was carried out at 37 °C and was initiated by the addition of 0.1 mL of specimen. For the precision and correlation studies, we used the Cobas-Bio with the same concentration of each reagent in the assay mixture as described above, but adding 30 μL of sample to 330 μL of the reagent mixture. The first measurement of absorbance was made at 180 s after the addition of sample, and again every 30 s thereafter for 300 s.

**Results**

**Synthesis of G7F.** The reaction mixture of cycloamaldextrin glucanotransferase with sucrose and β-cyclo-dextrin (22 h at 37 °C) was applied to the HPLC system to separate G7F from various fructosyl oligosaccharides (G2F, G3F, G4F, G5F, and G7F) as described in Materials and Methods, and a product with a retention time of 180 min was obtained.

To determine whether this product was G7F, we conducted the following tests: to 1.0 mL of the peak product we added 20 μmol/L phosphate buffer, pH 7.0, and 180 U of α-glucosidase in a volume of 2.0 mL, and incubated the mixture at 37 °C for 1 h. The reaction products were separated by HPLC with the above system, and only fructose and glucose were observed (the fructose:glucose ratio was 1:7). The product failed to react in Fehling’s test. For further study, we conducted ¹³C NMR analysis of the product. The result is shown in Table 1. The optical rotation of the peak product at 1.1 g/L in distilled water was [α]D 20.4 = +198.3. From these results we conclude that the peak product was G7F, as expected. The yield of G7F was about 12%, based on the quantity of β-cyclodextrin used.

**Synthesis of IPG7F.** IPG7F was synthesized from G7F as described in Materials and Methods. We analyzed the reaction products by TLC. Four spots (R value 0.07, 0.15, 0.25, and 0.35) were observed. The one with R = 0.07 was G7F. The product with R = 0.15 was considered to be IPG7F, which we purified by the preparative HPLC procedure described in Materials and Methods. The retention time of IPG7F was 36 min. To determine whether the product with the R = 0.15 was truly IPG7F, we conducted the following tests: optical rota-
tion, elemental analysis, $^{1}H$ NMR, and $^{13}C$ NMR. The optical rotation of a 1.0 g/L aqueous solution of product was $[\alpha]^{20}_{D} = +159.8$. By elemental analysis, the substance consisted of C = 45.09 and H = 6.51 (calculated: C = 45.2, H = 6.4). The $^{1}H$ NMR (D$_2$O; 270 MHz) showed peaks for seven anomeric protons at 5.46 to 5.38 ppm (which were situated only at the C-1 position of each glucose of IPG7F), those for the protons in the glucose ring at 4.05 to 3.63 ppm, and those for two methyl protons from the isopropylidene group at 1.55 and 1.42 ppm. The integral ratio of anomeric protons to methyl protons was 7:6. To further evaluate the structure of the product, we analyzed G7F, sucrose, maltoheptaose, and the product by $^{13}C$ NMR. The results of the analysis are also tabulated in Table 1. From the results of these studies, we concluded that the product was IPG7F. The yield was about 30%, based on the quantity of G7F used.

**Time course.** Figure 1A shows the relation of absorbance at 340 nm to time when $\alpha$-glucosidase, glucoamylase, and AMY were added sequentially to the mixture containing IPG7F, NADH, mannitol dehydrogenase, NaCl, CaCl$_2$, and PIPES buffer. Apart from a small drop in absorbance because of dilution, we observed no change in absorbance before the addition of these enzymes or after the addition of $\alpha$-glucosidase or glucoamylase. But the absorbance decreased in an apparent linear fashion after a short lag phase when human pancreatic AMY was finally added to the mixture.

**Table 1. $^{13}C$ NMR Spectral Data for Various Saccharides**

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<th>IPG7F</th>
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Acetyl-C | 30.7(ax)$^a$ |
Methyl-C | 21.2(eq)$^f$ |

$^a$3-(Trimethylsilyl)propionic acid d$_4$, sodium salt, as the internal standard, 270 MHz.
$^b$The number of carbon in the glucose group.
Sucrose: [G-1] \rightarrow 2[F].
$^d$Fructose.
$^e$Axial carbon (perpendicular to the acetal moiety).
$^f$Equatorial carbon (parallel to the acetal moiety).
Figure 1B shows the relation of absorbance to time when glucose, maltose, IPG7F, and intermediates such as G5F, sucrose, and fructose were added to the reaction mixture (AMY, NADH, PIPES buffer, and coupling enzymes such as α-glucosidase, glucoamylase, and mannitol dehydrogenase). When 100 μL of 100 mmol/L glucose solution was added to 2.7 mL of the reaction mixture, no absorbance change was observed. When 100 μL of 100 mmol/L maltose solution was then added, the absorbance was still not changed. However, when 100 μL of 30 mmol/L solutions of various reaction intermediates such as sucrose, fructose, and G5F were added to the mixture, the absorbance decreased rapidly. But when 100 μL of 120 mmol/L IPG7F reagent was added, we observed a linear change in absorbance, ascribed to AMY activity, after a brief lag phase.

Relation between the concentration of IPG7F and AMY activity. Figure 2 shows the relationship between the concentration of IPG7F and AMY activity. The greatest activities of pancreatic AMY and salivary AMY were obtained at the 4 mmol/L concentration of IPG7F. $K_v$ values, calculated by Lineweaver–Burk plot (15), were $1.4 \times 10^{-3}$ mol/L for pancreatic AMY and $9.5 \times 10^{-4}$ mol/L for salivary AMY.

Linearity. To determine the maximum AMY activity and the reaction rates of pancreatic AMY and salivary AMY with IPG7F, we measured the activity of pancreatic AMY and salivary AMY in various dilutions of the solution. We obtained straight lines that intercepted the origin (Figure 3).

Precision. Within-run and between-run variations were measured by use of two types of control sera with the Cobas-Bio centrifugal analyzer according to the method described in Materials and Methods. Within-run CVs were 1.0% (148 U/L) and 4.1% (47.8 U/L) and between-run CVs were 2.6% (145 U/L) and 5.3% (46.2 U/L).

Lack of interference by glucose and maltose. There was no interference with the enzyme reaction by glucose ≤40 g/L or by maltose ≤4 g/L.

Correlations. Figure 4 shows the correlation between the present method and the G5–hexokinase method or the G7pNP method. Although the number of samples was small (n = 46), the correlation coefficients were high and favorable results were obtained.

Discussion

When β-cyclodextrin, sucrose, and cyclomaltodextrin glucanotransferase were mixed, and the reaction products were separated by HPLC, we obtained several peaks. Judging from the data of Okada et al. (16), who isolated a coupling sugar, the substance with a retention time of 180 min appeared to be the desired product, G7F. Therefore, we collected this fraction. To determine whether the substance in this fraction was indeed G7F, we added a large amount of α-glucosidase to the material and incubated it for 1 h, and analyzed the reaction products by HPLC. The ratio of glucose/fructose was 7:1. Because the Fehling’s reaction was negative, no substance with a reducing end was present in the material. The results, accordingly, suggest that fructose is bound at the reducing end of the maltoheptaose.

The reaction mixture used in synthesizing isopropylidene G7F showed four spots on TLC. The substance with $R_f$ 0.07 was considered to be G7F; the others, with $R_f$s of 0.15, 0.25, and 0.35, were thought to be mono-, di-, and tri-isopropylidene derivatives of G7F, respectively.
We determined the exact structure of the product (36 min retention time on HPLC, 0.15 \( R_f \) on TLC) as follows: first, the product was found to be mono-isopropylidene G7F by elemental analysis and by the \( ^1H \) NMR spectra, in which the ratio of anomic to methyl protons was 7:6. Second, the isopropylidene group was considered to be bound across carbons 4 and 6 of the glucose at the nonreducing end of G7F according to the \( ^13C \) NMR spectra, which contained signals for C-4 and C-6 of the glucose at the nonreducing end at lower magnetic fields of 7.2 and 4.0 ppm, whereas signals at C-5 appeared at a higher magnetic field, 2.5 ppm, than those of G7F. Third, the structure of the isopropylidene group was determined to be in the chair form of the six-membered ring because the signals of the methyl carbons appeared at 30.7 and 21.2 ppm.

We investigated, by changing the order of addition of reagents or by adding an intermediate reaction product, whether any reaction occurs in the blank reaction and at what rate the intermediate product reacts. The results showed no change of absorbance when each of the reagents used was serially added, one by one, to mixtures containing IPG7F as a substrate (Figure 1A). In contrast, NADH was oxidized rapidly by the addition of glucoamylase in a similar experiment involving G5F with no modified nonreducing end (Figure 1B).

These results support three conclusions. First, IPG7F does not react with a coupling enzyme in the reaction mixture. By isopropylidenization of substrates, the hydrolysis by coupling enzymes, e.g., glucoamylase and \( \alpha \)-glucosidase, may be avoided because these enzymes can recognize the nonreducing end and hydrolyze. In particular, the addition of glucoamylase makes possible the use of maltohexaose or oligosaccharides with a longer chain for the determination of AMY, because comparatively long-chain reaction products, with which \( \alpha \)-glucosidase could not react, were rapidly hydrolyzed and detected. Second, multiple attack by AMY may not occur. Although the data are not shown in this paper, IPG7F was hydrolyzed by both isoenzymes and produced four products, G5F, G4F, G3F, and G2F. For the quick hydrolysis of these products by coupling enzymes, AMY does not react with them. Third, the change of NADH oxidation can be expressed as the exact activity. Even if there is a difference in hydrolysis site, the oxidation of NADH shows that exactly one molecule of NADH can be oxidized by one attack of AMY.

Furthermore, we had to choose an oligosaccharide with a longer chain than maltoheptaose when we used these substrates modified at both ends. Because AMY recognizes and reacts with the glucose with the modified group by using the shorter-chain substrates than maltopentaose modified at both ends, the AMY reaction cannot avoid the interference by these modified chemical groups. As reported elsewhere, AMY recognizes and reacts with five glucose molecules (18, 19).

When an intermediate product such as sucrose or fructose is added, we observed a time-course change similar to that of G5F (Figure 1B). The presence of a product other than G5F affects the oxidation of NADH. Unexpected reactions may not occur between reagents and AMY if there is no reaction in a specimen containing sucrose or fructose. Also, the assay of AMY by this system is not interfered with by these substances, because these substances react quickly with the coupling enzymes in the system.

\( K_m \) values of both isoenzymes for IPG7F were almost equal; therefore, both isoenzymes may hydrolyze IPG7F at almost the same reaction rate (Figures 2 and 3). Although the activities of both isoenzyme solutions were adjusted to be equal, small differences occurred in the reaction rates of both isoenzymes in the G5–hexokinase method because of a slight difference in the reaction rates for each isoenzyme (Figures 2 and 3).

The linearity in the range of the reagent concentrations tested in this study, for both isoenzymes ≤600 U/L, shows that AMY activity toward IPG7F was 1.5 times that of G5 (Figure 3). Also, in the G5–hexokinase
method, five molecules of NADH can be oxidized by one attack of AMY, whereas in the IPG7F method, only one molecule of NADH is oxidized by one attack. The sensitivity of the IPG7F method thus becomes 0.3 (i.e., 1.5/5.0) times that of the G5 method.

Reproducibility studies with two kinds of control sera yielded favorable results, with a CV of \( \leq 5\% \). In developing this determination method, it was most important to avoid any interference by various components of the specimens. Added glucose and maltose to AMY solution exerted no interference at \( \leq 40 \) g/L and 4 g/L, respectively.

From 46 subjects, we obtained favorable correlations of the G7pNP method and the G5–hexokinase method with our new method.

Many reports indicate that it is difficult to measure pNP because the appropriate pH value for AMY action is about 7.0 (6–8). As an extreme example, the absorbance of pNP doubles in the presence of \( \alpha \)-cyclodextrin because the solubility increases and the pH of the complex becomes more alkaline because of the hydroxyl groups of \( \alpha \)-cyclodextrin. Recently, various inventions have been developed to give easy administration or extend the effective period of medication by complexing undissolved drugs. But we must consider the influence of such complexed compounds on the determination of pNP. It has also been reported that accurately measuring pNP is difficult in the presence of proteins or free fatty acids, which shift the absorbance peak (20). Such interference will not occur when the oxidation rate of NADH is used for the determination, as was proved in glucose determinations with hexokinase. pNP has a sharp spectrum band, whereas NADH has a broad one. Determining a product with a sharp spectrum band requires a narrow spectrophotometric beam with a high degree of accuracy. Depending on the accuracy of the narrow beam, which depends on the instrument used, the result for pNP will change, whereas that for the oxidation of NADH will not. These results suggest that our method for assay of AMY can be used advantageously in the clinical laboratory, combining convenience, simplicity, and rapid results.

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