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AN IMPROVED ASSAY METHOD FOR AMYLASE ACTIVITY USING AN AMYLODEXTRIN FRACTION AS SUBSTRATE*

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ABSTRACT

A rapid and simple method for determination of amylase activity, by using a fraction of an amylo-oligosaccharide (amylodextrin, **1**) as substrate, is described. The sample solution is incubated with a solution of **1** and the substrate consumed is estimated by measuring the difference in absorbance at 460 nm. The K_m value of **1** is about half that of starch. The homogeneity of **1** in its chemical structure and molecular weight facilitated the specification of amylase units according to the international definition. This procedure was applied for assay of human-serum amylase with excellent reproducibility. This method did not require a large dilution factor, as the standard curve showed a linear relationship over a wide range of amylase concentrations.

INTRODUCTION

Numerous methods have been proposed for assay of alpha amylase [(1 → 4)-α-D-glucan 4-glucanohydrolase, EC 3.2.1.1], as it is of great help in the diagnosis of several diseases, such as acute pancreatitis[1]. These methods are usually grouped into two classes, saccharogenic[2] and amyloclastic[3], and the latter method has been used more widely as it is not affected by saccharides of low molecular weight. The primary problem of the amyloclastic method, however, lies in the starch substrate itself[4]. The starch is heterogeneous as regards molecular weight and also degree of branching, and these factors influence the susceptibility of the starch to amylolytic attack. Amylose, being less branched than starch, has been employed[5], but the reproducibility is, nevertheless, still affected by variations in its molecular weight.

This paper describes an assay method for amylase activity based on an amylo-oligosaccharide fraction (amylodextrin, **1**)[6,7] as a substrate of homogeneous chemical structure.

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*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

EXPERIMENTAL

General methods. — The following human sera containing amylases of various activities were used for reference: Multi-Enzyme Reference Serum (MERS), Hyland Div. Travenol Laboratories, Inc., and Consera® and Consera A®, Nissui Seiyaku Co. The substrate solution was prepared by dissolving 10.0 mg of **1** (5.10 μmol, assuming the molecular weight of **1** to be 1962) in 10 ml of 0.1M phosphate buffer (pH 7.0), and maltotriose solution was prepared by dissolving 10.3 mg of maltotriose (20.4 μmol) in 10 ml of the same buffer. Iodine reagent contained 8.3mM iodine, 42mM potassium iodide, and 0.23mM hydrochloric acid. Absorption spectra and absorbances were recorded with a Hitachi Model 200–20 spectrophotometer.

Assay procedure. Three test tubes (A, B, and C) are used for each sample. Into tubes A and B is pipetted 0.5 ml of the substrate solution and into tube C, 0.5 ml of maltotriose solution. The three tubes are preincubated for 3 min at 37°. Into each of tubes A and C is pipetted 0.1 ml of the sample. All of the tubes are incubated again for 3 min at 37°. To each tube is added 6 ml of iodine reagent, and the tubes are shaken vigorously. Into tube B is then added 0.1 ml of the sample solution. Finally, the absorbances are measured at 460 nm. Duplicate or triplicate runs are recommended for each sample to assure accuracy.

Calculation of the amylase unit. One unit of amylase is herein defined as that activity which hydrolyzes 1 μmol of **1** in 1 min, giving a hydrolyzate that does not form a colored complex with iodine. Consequently, the unit is calculated as follows:

$$\frac{b-a}{b-c} \times f = \text{unit/l} \qquad \text{(Eq. 1)}$$

where a, b, and c indicate the absorbances shown by the mixtures in tubes A, B, and C, respectively, and

f = μmol of **1** present in the mixture ×

$$\times \frac{1000 \text{ ml}}{\text{volume of the sample solution in ml}} \times \frac{1}{3} = 0.255 \times \frac{1000}{0.1} \times \frac{1}{3} = 850$$

This definition of the unit is used throughout the present paper, unless otherwise stated.

High-speed liquid chromatography. An HLC-802R high-speed liquid chromatography apparatus (Toyo Soda Manufacturing Co., Ltd.) was used for analysis of amylodextrins under the following conditions. Columns, two TSK-GEL-G 2000 SW columns, 7.5-mm diameter, 60-cm long; eluant, a mixture of 0.067M potassium dihydrogenphosphate (2 parts) and 0.067M disodium hydrogenphosphate (8 parts), pH 7.4; flow rate, 0.8 ml/min; pressure, 60 kg/cm^2; temperature, 40°; detector, refractive index; sample size, 200 μl of 2% solution.

Preparation and characterization of **1** (ref. 6). Nägeli amylodextrin was first prepared by an adaptation of the procedure of Kainuma and French[7]. Sulfuric acid (16%, 1.1 liters) was added to waxy corn starch (30 g) in a capped, plastic bottle.

This mixture was kept for 50 days at 37° with occasional shaking. Every ten days, the sulfuric acid was replaced by fresh acid by decantation. The degree of hydrolysis of waxy corn-starch virtually reached equilibrium (70%) after 40 days of this treatment. Residual, water-insoluble carbohydrate was washed with large amount of cold water, sufficient to remove sulfuric acid and other water-soluble substances. After washing, the excess of water was removed as thoroughly as possible by centrifugation. Methanol was added to the residual amylodextrin until it showed no stickiness, and the excess of methanol was filtered off by vacuum filtration. The residue was washed with abs. methanol and 1-butanol, and then dried at room temperature and atmospheric pressure. About 8 g of Nägeli amylodextrin was obtained. The resulting native amylodextrin consists of two comparatively homogeneous components, Fraction II and Fraction III, as shown in Fig. 1. Each fraction was separated by the following procedure: native amylodextrin (5 g) was dissolved in boiling, 50% pyridine (100 ml). After cooling to 50°, methanol (300 ml) was added to the solution and this mixture was kept overnight at room temperature with gentle stirring. The precipitate of higher molecular weight (Fraction II) was separated by centrifugation, washed with the same solvent mixture and abs. methanol, and dried at room temperature and atmospheric pressure. The supernatant was evaporated to dryness under diminished pressure to recover Fraction III. Yields of Fractions II and III were 3.3 g and 1.4 g, respectively. The profile of gel-permeation chromatography by high-speed liquid chromatography is shown in Fig. 1. Fraction III consisted mainly of linear malto-oligosaccharides whose reducing values indicate an average molecular size of about 12 D-glucose residues. Fraction II, d.p. ~25, was primarily singly branched at C-6, with the branching point near the reducing group. Only Fraction III was used for the amylase assay in this report.

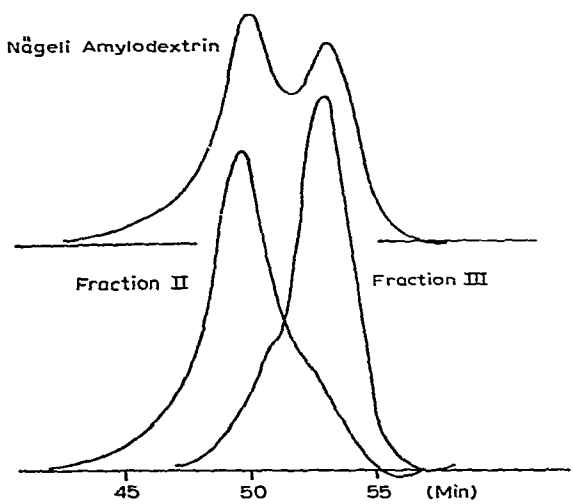

Fig. 1. Gel-permeation chromatography of Nägeli amylodextrin, Fraction II and Fraction III.

RESULTS

Fig. 2 illustrates the difference spectrum between the blank and the sample containing amylase. The maximal difference was observed at 460 nm, and this wavelength was chosen for the standard procedure. Fig. 3 displays the Lineweaver–Burk plot of the system containing human-serum amylase and **1**. A linear relationship was observed in the range of $1/S$ of 2.35mM^{-1} to 11.8mM^{-1}, and a K_m value of 141μM was obtained from the data. Accordingly, the substrate concentration of 425μM in the incubation mixture (that is, 510μM of **1** in the substrate solution) was adopted in the standard procedure.

Although the mixture of completely hydrolyzed substrate and the enzyme gave an absorbance a little lower than the enzyme alone on addition of iodine reagent, complete saponification of **1** required a high activity of amylase and prolonged

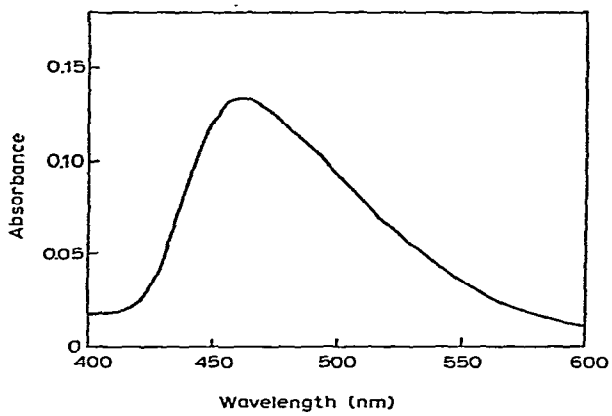

Fig. 2. Difference spectrum between the sample of human-serum amylase (MERS, 132 unit/l) and the blank.

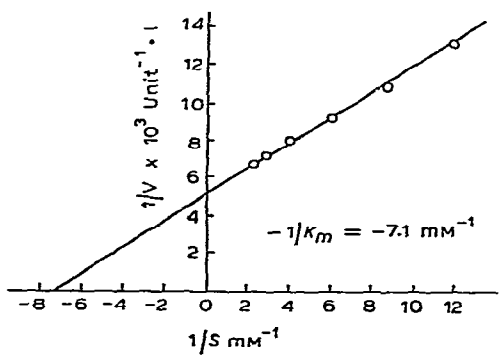

Fig. 3. Lineweaver–Burk plot of the system containing human serum amylase (MERS, 156 unit/l) and **1**.

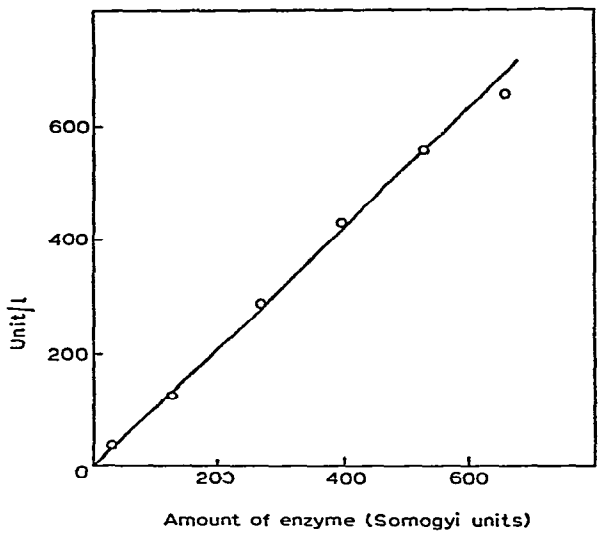

Fig. 4. Standard curve for human-serum amylase (MERS).

TABLE I

AMYLASE ACTIVITIES (UNITS/LITER) OF HUMAN SERUM, AS ESTIMATED BY THE PRESENT METHOD

No. of assay	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*Average*	*C.v.* (%)
Serum 1[a]	154	151	149	151	153	152	155	152	1.24
Serum 2[b]	357	359	356	357	356	359	355	357	0.40
Mixed serum[c]	219	216	215	218	219	220	220	218	0.83

[a]Consera, 128 Somogyi unit. [b]Consera A, 324 Somogyi unit. [(Units measured by the amylase test, Daiichi Kagaku Yakuhin Co. Ltd. (Tokyo)]. [c]Sera 1 and 2 were mixed 2:1 by volume.

reaction. Maltotriose solution was used in place of the complete hydrolyzate of **1** in calculation of the unit (Eq. *1*), as the former gave the same absorbance as latter.

The standard curve for human-serum amylase (Fig. 4) showed a linear relationship in the range 30–500 Somogyi units of amylase, and passed through the origin.

Table I summarizes the results of assay of human-serum amylase. The human serum having normal amylase activity (serum 1) and that of abnormally high amylase activity (serum 2) showed average activities of 152 and 357 unit/l, respectively, and coefficients of variation (c.v.) of 1.24 and 0.40%, respectively.

The sample prepared by mixing 2 volumes of serum 1 and 1 volume of serum 2 gave an average activity of 218 units/liter, and the coefficient of variation was 0.83%. The observed unit was in good agreement with the unit/liter of 220 calculated from the component serum. These results indicate that the present method is useful for the determination of serum amylase.

DISCUSSION

As already mentioned in the introduction, the use of homogeneous, unbranched saccharides has long been expected to solve the problem of amylase assay. In addition, existing amyloclastic methods using iodine[3,5] were too sensitive for the estimation of serum or pancreatic fluid, and dilution by a large factor often caused serious error.

Compound **1**, having excellent homogeneity and moderate reactivity with iodine, seemed to be suitable for this purpose. It showed a difference spectrum giving a distinct maximum at 460 nm (Fig. 2) on reaction with iodine.

The molecular-weight homogeneity of **1** facilitated expression of the amylase unit according to the international definition[8], whereas numerous particular definitions of amylase unit have hitherto been proposed, because of the heterogeneity of polysaccharide substrates. In addition, the values of the unit obtained by the present method were in good agreement with those by the Somogyi method (Fig. 4). Accordingly, the data given by the two methods may readily be compared.

The Lineweaver–Burk plot (Fig. 3) demonstrated that the K_m value of this substrate was 141 μM, that is, 277 μg/ml. This value is about half of the K_m value reported for starch[9]. In the standard procedure, a rather low concentration of **1** ($\sim 3\ K_m$) was used in order to keep the absorbance within such a range that dilution of the reaction mixture might be minimized. Incubation of the substrate with the enzyme was carried out in a volume as small as 0.6 ml. This procedure diminished both the pre-incubation time and the amount of **1** used. Consequently, the present procedure required a total incubation time of only 6 min.

A standard curve for human-serum amylase, based on **1**, was found to remain linear up to significantly higher amylase activity as compared with that using starch[4]. Therefore, this method does not require the large dilution-factor essential for the methods employing starch substrate. Smaller volumes of serum (such as 20 μl) may be used for the measurement of extremely high activity.

Application of this technique to the assay of serum amylase (Table I) proved its excellent reproducibility. A mixture of normal and abnormal serum showed satisfactory recovery of the activity.

The present method provides a rapid and simple tool for the assay of amylase. The amylase unit is expressed according to the international definition. This method may be promising for the automated analysis of biological fluids, as it does not require a large dilution factor.

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