Applicability of a New Synthetic Dye-Labeled Substrate for Amylase Assay[1] (34656)

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Although measurement of amylase activity in the serum and urine is a valuable clinical tool, present methods of assay are relatively complex, and have certain deficiencies (1). Techniques using substrates composed of polysaccharides covalently bound to a dye have recently been advanced as simple and convenient methods for amylase assay (2, 3). When these substrates are acted on by amylase, fragments are released. The remaining unhydrolyzed substrate is precipitated by centrifugation and the depth of color in the supernatant containing the released fragments taken as the measure of amylase activity.

Studies conducted by us (4, 5) on a new dye-labeled substrate, Cibachron blue F3GA–amylose (6, 7), indicated that amylase assays obtained with this compound compare favorably with those using an established saccharogenic method. Nevertheless, it seemed of value to obtain supporting evidence that the hydrolysis of Cibachron blue F3GA–amylose (CBA) is the result solely of amylase activity. Accordingly, the enzymatic hydrolysis of CBA by human serum and saliva was compared with that by crystalline amylase. The results support the interpretation that the enzymatic activity measured by hydrolysis of CBA is that of amylase.

Methods and Materials. The synthesis of CBA and information concerning its structure have been described by Klein *et al.* (6, 7). CBA is very slightly soluble in water. This is advantageous when the substrate is clinically employed to assay amylase activity (4–7) because the unhydrolyzed CBA can easily be separated from the colored water-soluble fragments. However, control and definition of substrate concentration is not possible under these conditions. To obtain the enzymological data we sought, it was necessary to define and reproducibly vary the CBA concentration. This could only be achieved by solubilizing the CBA.

To prepare an aqueous solution, 200 mg of CBA were placed in an 18 × 150-mm test tube and 1.6 ml of dimethylsulfoxide (DMSO)[2] were added. The mixture was heated to 70–75° and then periodically was stirred with a glass rod over a period of 10 min. The glass rod was used with a grinding motion against the walls of the test tube. Another 0.2 ml of DMSO was next added and stirring was continued for another 15 min. An additional 0.2 ml of DMSO was then used to remove the CBA from the sides of the test tube and the mixture was stirred occasionally for another 30 min. To the homogeneous, viscous solution resulting from this treatment, 18 ml of distilled water (at a temp of 50°) were added slowly and stirring was continued until a fully homogeneous solution was obtained. The reagent so prepared represented a 1% aqueous solution of CBA containing 10% DMSO. This solution was stable for several weeks at room temperature, but became cloudy if refrigerated. The stock solution was used either as prepared, or after the addition of aqueous 10% DMSO. This yielded solutions of fixed DMSO concentration, but with varying concentrations of CBA. All optical density measurements were carried out with a Beckman DU spectrophotometer using a light path of 1 cm.

Two methods were used to measure enzy-

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[2] Reagent Grade, Fisher Scientific Co., Fairlawn, N. J.

matic digestion of CBA: an ethanolic-trichloracetic acid (TCA) method, and an iodometric method. With the ethanolic-TCA precipitation method, an incubation mixture was prepared by mixing 0.1 ml of enzyme source (human saliva, human serum, or hog pancreatic α-amylase)[3], 0.3 ml of buffered saline (0.2 *M* sodium phosphate at pH 6.9 in 0.154 *M* NaCl), and 0.4 ml of aqueous CBA solution containing 10% DMSO. After incubation at 37° the reaction was stopped by adding 2 ml of absolute ethanol containing 10% TCA. After refrigeration, the material was centrifuged (3000 rpm for 10 min) in a refrigerated centrifuge at 4°. Optical density of the supernatant containing the soluble colored products was measured at 625 mμ.

The other method used to measure enzymatic hydrolysis of CBA was an iodometric technique based on that of Street and Close (8). The enzyme sources were prepared as follows: Hog pancreas α-amylase suspension (30,000 IU/ml) was diluted 1000-fold with 0.05 *M* sodium phosphate (pH 6.9) in 0.036 *M* NaCl. Serum from a patient with acute pancreatitis having an amylase level of 3890 Somogyi units was diluted 200-fold with the same buffer. Saliva was diluted with sufficient of the identical buffer to reduce the amylase activity to a level approximating that of the diluted serum. The procedure was as follows: A mixture was prepared composed of 1.0 ml of enzyme source, 0.5 ml of water, and 0.5 ml of CBA solution in 10% DMSO. During incubation at 37°, samples were taken at 3 points in time to construct a slope representing the rate of disappearance of the CBA as evidenced by reduction in intensity of the colored complex formed between CBA and iodine. Iodometric determinations of CBA were made in duplicate on 0.3-ml samples that were transferred to an iodine solution (0.005% iodine in 0.167% KI). When the final concentration of CBA in the incubation mixture was 0.031%, the 0.3-ml sample was added to 3.0 ml of iodine solution; at higher concentrations of CBA, the size of the sample was kept at 0.3 ml, but the volume of iodine solution was increased in proportion to the increase in CBA concentration. Optical densities were read at 620 mμ. The dye moiety in CBA did not appreciably interfere with the iodometric assay of CBA disappearance. This was evidenced by measurements of optical density carried out with, and without, iodine which indicated that the absorbance due to the dye moiety in CBA is only 2.1% of the absorbance due to the CBA–iodine complex.

The effects of maltose, sucrose, rabbit liver glycogen[3], and shellfish glycogen[3] on amylase activity measured by CBA were determined by both the iodometric and ethanolic-TCA precipitation methods. These substances were dissolved in water when the former method was used, and in buffered saline when the latter method was employed. The amounts of dissolved substances added yielded final concentrations in the incubation mixture of 0.1 *M* maltose, 0.1 *M* sucrose, 0.125% rabbit liver glycogen, and 0.125% shellfish glycogen, respectively.

To test for chromogenicity of CBA with iodine, the following were added to a cuvette: 1 ml of the buffered saline; 0.5 ml of 0.02% CBA in 10% DMSO; 0.15 ml of 0.112% iodine in 0.4% potassium iodide; and 1.35 ml of distilled water. In another experiment, amylose with a degree of polymerization of about 300[4] was substituted for CBA.

The effects of DMSO on amylase activity were tested by means of the iodometric method using soluble starch[5] instead of CBA as substrate.

All experiments were conducted twice, each time in duplicate.

Results. The data given in Table I were obtained with the use of the ethanolic-TCA precipitation method. The amount of soluble colored products found in the supernatant was reduced as the concentration of chromogenic substrate was increased. This appeared to indicate enzyme inhibition by the substrate. However, the addition of more unhydrolyzed CBA solution to the hydrolytically-released products immediately before addition of the ethanolic-TCA further lowered the amount of colored products found in the supernatant.

[3] Mann Research Laboratories, Orangeburg, N. Y.

[4] Nutritional Biochemicals, Cleveland, Ohio.

[5] Merck and Co., Rahway, N. J.

TABLE I. Effects of Increasing CBA Concentration on Hydrolysis of CBA by Crystalline Amylase and Human Serum and Saliva (Ethanolic-TCA Precipitation Method of Assay).

CBA conc (%)	Optical density (625 mμ) of centrifuged supernatant		
	Hog pancreatic α-amylase[a]	Human serum[b]	Human saliva[c]
0.063	0.078	0.066	0.070
0.125	0.036	0.024	0.039
0.25	0.020	0.012	0.020
0.50	0.014	0.008	0.012

[a] 35-sec incubation.
[b] 4-min incubation.
[c] 6-min incubation.

An inhibitory effect of increasing CBA concentration was not found when the experiment was repeated with crystalline amylase using the iodometric technique (Table II).

The observations made using the iodometric method indicate that 0.1 *M* maltose, in contrast to sucrose, markedly inhibited the three sources of amylase tested (74 to 79% inhibition). The effects obtained with serum and saliva paralleled those obtained with crystalline α-amylase (Table III). Glycogen also displayed an inhibitory effect on crystalline α-amylase (Table IV). More pronounced effects were observed, however, with serum. Similar results were obtained using the ethanolic-TCA precipitation method.

Enzymatic hydrolysis of CBA occurred at a linear rate (Fig. 1). As shown in Fig. 1, 0.1 *M* maltose exerted a 38% inhibition of serum activity at a CBA concentration of 0.125%. CBA in solution formed a deep blue color with iodine which was about 57% as intense as that formed by the same mass of

TABLE II. Rate of CBA Hydrolysis at Various Substrate Concentrations in Presence of Hog Pancreatic α-Amylase (Iodometric Method of Assay).

CBA conc (%)	Disappearance of CBA (mg/ml/min)
0.031	0.288
0.063	0.308
0.125	0.377
0.250	0.307

TABLE III. Effects of Maltose and Sucrose on Amylase Activity (CBA Conc 0.031%) (Iodometric Method of Assay).

	CBA disappearance rate (mg/ml/min)		
	Hog α-amylase	Serum	Saliva
Control	0.439	0.020	0.023
0.1 *M* Maltose	0.101	0.005	0.005
0.1 *M* Sucrose	0.426	0.019	0.024

amylose. With soluble starch as the substrate, it was found that DMSO concentrations ranging from 2.5% to 12.5% did not inhibit amylase activity.

Discussion. The data dealing with the effect of increasing CBA concentration (Table I) would suggest that CBA inhibits its own hydrolysis. However, when unhydrolyzed CBA was added to the hydrolyzed material and ethanolic-TCA added, coprecipitation of some of the colored products apparently resulted. Thus, the effect noted at higher CBA concentrations was not due to inhibition by substrate, as we had first suspected (9), but to coprecipitation of hydrolytically released fragments by unhydrolyzed CBA. Since such precipitation does not occur with the iodometric method, there was no problem arising from coprecipitation of hydrolytic products when this method was employed. Use of the iodometric method confirmed that substrate inhibition did not occur (Table II).

Schwimmer (10) found that maltose was a noncompetitive inhibitor of malt α-amylase. Further, Whitaker *et al.* (11) showed that

TABLE IV. Effects of Glycogen on Amylase Activity (CBA Conc 0.031%) (Iodometric Method of Assay).

	CBA disappearance rate (mg/ml/min)	
	Hog α-amylase	Serum
Control	0.439	0.014
Shellfish glycogen (0.125%)	0.231	0
Rabbit liver glycogen (0.125%)	0.220	0

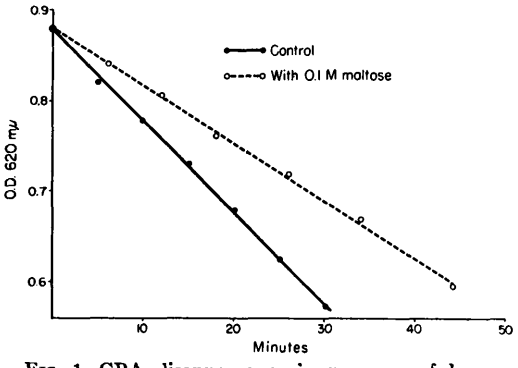

FIG. 1. CBA disappearance in presence of human serum as measured iodometrically (initial CBA conc 0.125%).

structural factors are important in determining the effects of additives on hog pancreas α-amylase activity. They found that maltose was more inhibitory than sucrose and lactose. Since maltose is a product of amylase action, it may be bound to the active site of the enzyme at some stage of the reaction and, at sufficiently high concentration, could be inhibitory. Our data and those of Whitaker *et al.* (10) appear to be consistent with this hypothesis.

That both types of glycogen tested were also inhibitory is probably due to binding of the enzymes by these amylase substrates. The lesser inhibitory effect by the glycogens on crystalline amylase than on serum amylase may be a secondary effect related to the fact that the amylase activity in the experiments with crystalline material was far greater than in those with serum. In the procedure employed, a brief period of about 1 or 2 min elapsed before CBA was added. Amylase could conceivably have acted on the glycogen during that time. Thus, at very high amylase activity, the glycogen may have been degraded to products having lesser inhibitory power than the original glycogen.

Of note was the fact that the inhibitory effect of maltose on serum amylase activity was not as great at a higher CBA concentration. This raises the speculation that, in contrast to malt α-amylase, the human serum enzyme may be inhibited in a competitive manner by maltose.

That CBA develops a colored complex with iodine indicates that CBA forms a helical complex with iodine similar to amylose. Because of the dye molecules attached to it, CBA probably has a greater mass per glucose unit than amylose. The two compounds, therefore, are probably quantitatively similar with regard to their iodine chromogenicity. Furthermore, it would seem that the addition of dye moieties to the carbohydrate molecule does not radically alter the conformational characteristics that determine the reaction with iodine. These properties may be important in the enzyme–substrate interaction.

The foregoing observations lend additional support to the conclusion that CBA hydrolysis represents amylase activity under the conditions employed. It should be emphasized that for the purposes of these studies of the enzymatic hydrolysis of CBA, it was necessary to solubilize the substrate. For clinical use in assaying amylase activity, it is preferable to employ CBA in the form of an aqueous suspension (6, 7).

Summary. Observations were made on the hydrolytic effect of human serum and saliva, as well as hog pancreatic α-amylase, on a new chromogenic substrate, Cibachron blue F3GA-amylose (CBA). Maltose markedly inhibited hydrolysis of CBA while sucrose did not. This obtained whether the enzyme source was human serum, saliva, or crystalline α-amylase. Rabbit liver glycogen and shellfish glycogen also inhibited hydrolysis by either serum or crystalline amylase. The various findings were interpreted to indicate that hydrolysis of CBA reflects amylase activity.

The Cibachron blue F3GA–amylose was kindly prepared and supplied by Drs. Bernard Klein and Ronald L. Searcy, Department of Diagnostic Research, Hoffmann-La Roche Laboratories, Nutley, N. J.

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