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NEW CHROMOGENIC SUBSTRATES* FOR THE ASSAY OF ALPHA-AMYLASE AND (1→4)-β-D-GLUCANASE

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ABSTRACT

New chromogenic substrates have been developed for the quantitative assay of alpha-amylase and (1→4)-β-D-glucanase. These were prepared by chemically modifying amylose or cellulose before dyeing, to increase solubility. After dyeing, the substrates were either soluble or could be readily dispersed to form fine, gelatinous suspensions. Assays based on the use of these substrates are sensitive and highly specific for either alpha-amylase or (1→4)-β-D-glucanase. The method of preparation can also be applied to obtain substrates for other endo-hydrolases.

INTRODUCTION

The assay of polysaccharide endo-hydrolases has been greatly simplified over recent years by the introduction of assays employing dye-labelled (or chromogenic) substrates. Many substrates have been developed for the assay of alpha-amylase (EC 3.2.1.1)[1–4], because of its importance in clinical biochemistry and its involvement in pre-harvest sprouting in rain-damaged wheat[5]. However, most of the chromogenic substrates are insoluble and have the inherent disadvantages of heterogeneity in the assay tube and the difficulties associated with dispensing a solid substrate routinely with accuracy. Pre-weighed tablets of the chromogenic substrates are commercially available[6,7], but are costly. Soluble, dye-labelled amylopectin is also commercially available[8], but is very costly, possibly due in part to difficulties associated with removing unbound dye[3].

Cellulose Azure, which is the only commercially available, chromogenic substrate for the assay of (1→4)-β-D-glucanase (endo-cellulase), is insoluble and very resistant to enzymic hydrolysis. Huang and Tang[9] have reported techniques for the preparation of both a chromogenic and a fluorometric substrate for endo-(1→4)-β-D-glucanase. However, their preparation involves the use of expensive and toxic chemicals, and the chromogenic substrate is both unstable and insoluble.

Recently, a method was described for the preparation of carob galactomannan dyed with Remazolbrilliant Blue R as a substrate for the sensitive assay of β-D-mannanase[10]. The degree of galactose substitution on the mannan polymer was

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critical; it had to be sufficient to impart solubility, but not so high (*e.g.*, as in guar galactomannan) that it impaired the initial rate of hydrolysis of the mannan backbone by the β-D-mannanase. The degree of galactose substitution in carob galactomannan satisfied both these requirements.

It is now reported that similar, sensitive, chromogenic substrates can be prepared from cellulose and amylose for the assay of (1→4)-β-D-glucanase and alpha-amylase, respectively, if the polysaccharide is first chemically modified to increase solubility.

MATERIALS AND METHODS

Enzymes. — Porcine, pancreatic alpha-amylase (A 2643) was obtained from Sigma Chemical Co. (U.S.A.). (1→4)-β-D-Glucanase (cellulase) was purified from a commercial Driselase preparation (Kyowa, Hakko Kogyo Co. Ltd., Japan) as follows. The crude powder (10 g) was suspended in water (25 mL) with stirring and centrifuged (2,000*g*, 10 min) to remove insoluble material, and the supernatant solution was adjusted to pH 7, cooled to 2°, and dialyzed against ice-cold phosphate buffer (20mM, pH 7). The solution was applied to a column (2.5 × 80 cm) of Ultrogel AcA44 and eluted with the same buffer. Fractions rich in (1→4)-β-D-glucanase were combined, applied to a DEAE-Sepharose column (2.5 × 10 cm, pH 7), and eluted with a linear salt gradient (0→0.4M KCl; total volume, 1 L) in phosphate buffer (20mM, pH 7). The major peak of (1→4)-β-D-glucanase was eluted at ~0.1M KCl. This was concentrated and the pH adjusted to 3 by washing with sodium citrate buffer (20mM, pH 3), in a Diaflo ultrafiltration cell with a PM10 membrane. The solution was applied to a column (2.5 × 20 cm) of SP-Sephadex and eluted with a linear salt gradient (0→0.4M KCl; total volume, 1 L) in citrate buffer (20mM, pH 3). The fraction rich in (1→4)-β-D-glucanase was concentrated by ultrafiltration and the pH adjusted to 4.5 by washing with sodium acetate buffer (100mM, pH 4.5). (1→4)-β-D-Glucanase was purified 27-fold, to a specific activity of 835 nkat (nmol of D-glucose equivalents released from CM-cellulose 4M6S per second per mg of protein). The preparation was completely devoid of β-D-glucosidase activity.

A cellulase preparation (Sigma C7502) and crude, intestinal juice from *Helix pomatia* (Sigma GO876) were also used as sources of (1→4)-β-D-glucanase. Amyloglucosidase (Cat. No. 171581) and beta-amylase (Cat. No. 171577) were obtained from Calbiochem–Behring Corp. (U.S.A.).

Preparation of dyed CM-cellulose. — Commercially available *O*-(carboxymethyl)cellulose (CM-cellulose) [50 g, CMC-4M6SF, Hercules Inc. (U.S.A.)] was added with blending (Sorvall Omnimix) to a solution of cellulase (50 mg, Sigma C7502) in sodium acetate buffer (1 L, 0.1M, pH 5) during 30 min. The solution was then blended vigorously and incubated at 40° for 30 min, to allow partial depolymerization of the CM-cellulose. Remazolbrilliant Blue R (10 g) or Remazolbrilliant Black B (10 g, Hoechst Aust. Ltd.), potassium chloride (50 g), and trisodium phosphate (10 g) in water (50 mL) were then added with stirring. The temperature was

increased to 60° and the solution was stirred for 60 min. The solution was cooled, and unbound dye was removed by precipitation of the polysaccharide from solution by the addition of 1.5 vol. of ethanol containing KCl (0.02M). The CM-cellulose, to which the dye was covalently linked, was then washed by suspension in 60% aqueous ethanol containing KCl (0.02M) and collected on a fine sieve. This procedure was repeated twice, and then the dyed polymer was redissolved and reprecipitated until the washings were colorless (2–3 times).

Preparation of Remazolbrilliant Blue R (RBB) CM-amylose and CM-soluble starch[11,12]. — Corn or potato amylose (100 g) or soluble starch [100 g, Baker Analyzed (Lintner) soluble; J. T. Baker Chemical Co. (U.S.A.)] was added to a solution of chloroacetic acid (14.4 g) in ethanol (260 mL) and heated with stirring. When the solution started to boil, a solution of NaOH (60 mL, 8M) in ethanol (340 mL) was added dropwise during 15 min, and boiling under reflux was continued for 15 min. The solution was cooled and filtered, and the insoluble product was washed with 80% aqueous ethanol and then dissolved in boiling water (1 L). The solution was adjusted to 60° and a sample was taken for determination of the degree of carboxymethylation[11]. Remazolbrilliant Blue R (20 g), potassium chloride (50 g), and trisodium phosphate (10 g) were added, and the solution was stirred for 60 min, cooled, and added with stirring to 1.5 vol. of ethanol containing KCl (0.02M). The precipitate was collected on a fine sieve and washed as for the Remazolbrilliant Blue R CM-cellulose. When all free dye had been removed, the polysaccharide was dried by washing with ethanol and then acetone. The product had a d.s. of carboxymethyl groups of ~0.15 and a ratio[10] of Remazolbrilliant Blue R to "anhydrohexose" of ~1:50. CM-amylose with a d.s. of 0.2 was prepared by increasing the amounts of chloroacetic acid and NaOH used by ~33%. Solutions of this material and of CM-soluble starch (d.s., 0.1), when dyed with Remazolbrilliant Blue R, formed rubbery precipitates on addition to 1.5 vol. of ethanol. These precipitates were squeezed free of liquid, redissolved by blending in hot water, and reprecipitated. This procedure was repeated, to remove unbound dye.

Dissolution of substrates. — Powdered substrate (2 g) was dissolved in hot water (80 mL) with vigorous stirring, and sodium acetate buffer (5 mL; 2M, pH 5) or sodium phosphate buffer (5 mL; 2M, pH 6.9) was added. The solution was cooled and diluted to 100 mL after adjustment of the pH.

Assay of (1→4)-β-D-glucanase. — (*a*) *Reducing-sugar assay.* Enzyme preparation (0.1 mL) was incubated with a solution of CM-cellulose (CMC 4M6SF, 0.5 mL, 1%) in acetate buffer (0.1M, pH 5) for up to 10 min at 40°. The reaction was terminated by the addition of 4-hydroxybenzohydrazide[13] reagent (5 mL) and the color was developed by incubation for 6 min at 100°. One nkat of enzyme activity released one nmol of reducing-sugar equivalent (as glucose) per second at 40° and pH 5.

(*b*) *Chromogenic assays.* Enzyme preparation (0.1 mL) was incubated with dyed CM-cellulose (0.5 mL) in acetate buffer (0.1M, pH 5) for up to 10 min at 40°. The reaction was terminated by the addition of a precipitant solution (2.5 mL) that contained 80% ethylene glycol monomethyl ether, sodium acetate buffer (0.3M,

pH 5), and zinc acetate (0.4%). The solution was vortexed for 10 sec and centrifuged (2,000*g*, 5 min), and the absorbance of the supernatant was measured at 590 nm.

Enzyme activity on Cellulose Azure (Calbiochem Cat. No. 219481) was measured by incubating the enzyme solution (0.1 mL) with Cellulose Azure (10 mg) in acetate buffer (0.5 mL; 0.1M, pH 5) for up to 2 h in an oscillating incubator bath. The reaction was terminated by the addition of 0.12M NaOH (2.5 mL), and the solution was filtered prior to measurement of absorbance at 590 nm. The rate of hydrolysis of TNP-cellulose [*O*-(2,4,6-trinitrophenyl)cellulose] was determined by incubating enzyme solution (0.1 mL) with a 1% suspension of TNP-cellulose in sodium acetate buffer (pH 5, 0.1M) at 40°. At intervals, portions of the solution were filtered through glass-fibre filter papers, and the absorbance (344 nm) of the filtrate was measured (*cf.* Ref. 9).

Assay of alpha-amylase. — (*a*) *Reducing-sugar assay.* Enzyme preparation (0.1 mL) was incubated with soluble-starch substrate (0.5 mL, 10 mg/mL) in sodium glycerophosphate buffer (0.1M, pH 6.9) at 37°. The reducing sugar was then determined by the Nelson–Somogyi method[14,15].

(*b*) *Chromogenic assays.* Enzyme preparation (0.1 mL) was incubated with dyed CM-amylose or dyed CM-starch (0.5 mL, 2%) in phosphate buffer (0.1M pH 6.9) for up to 10 min at 40°. The reaction was terminated as in (*b*) above).

The rate of hydrolysis of RBB-amylose and Amylopectin Azure (Sigma A4640) was determined by incubating enzyme solution (0.1 mL) with substrate (0.5 mL, 2%) in phosphate buffer (0.1M, pH 6.9) for up to 10 min. The reaction was stopped by the addition of 1.2M NaOH (2.5 mL), the solution filtered, and the absorbance of the filtrate measured at 590 nm.

Enzyme action on commercial Phadebas® and Amylochrome® substrates[6,7] was determined by suspending the tablets in water (4 mL) and adding alpha-amylase solution (0.1 mL). After incubation for up to 10 min, the reaction was terminated by the addition of NaOH (0.1M, 1 mL). The solutions were filtered, and the absorbance was measured at 625 nm (Amylochrome) or 620 nm (Phadebas).

The assay of alpha-amylase activity on Dyamyl L[8] was performed essentially as described by the manufacturer, with the modification that all volumes were halved.

RESULTS AND DISCUSSION

In these studies, chromogenic substrates for the assay of alpha-amylase and (1→4)-β-D-glucanase (endo-cellulase) have been prepared by dyeing cellulose and amylose which had been solubilized by substitution with carboxymethyl groups. The optimal d.s. to impart solubility without seriously affecting the susceptibility of the polysaccharide to enzymic hydrolysis was between 0.1 and 0.4. At a d.s. <0.2, the polysaccharides were rendered partially insoluble upon dyeing. At a CM-d.s. approaching 0.7, the dyed polysaccharides were quite resistant to enzymic attack and hence the sensitivity of the assay was greatly diminished.

The ionic strength of the substrate, enzyme, and precipitant solutions affected

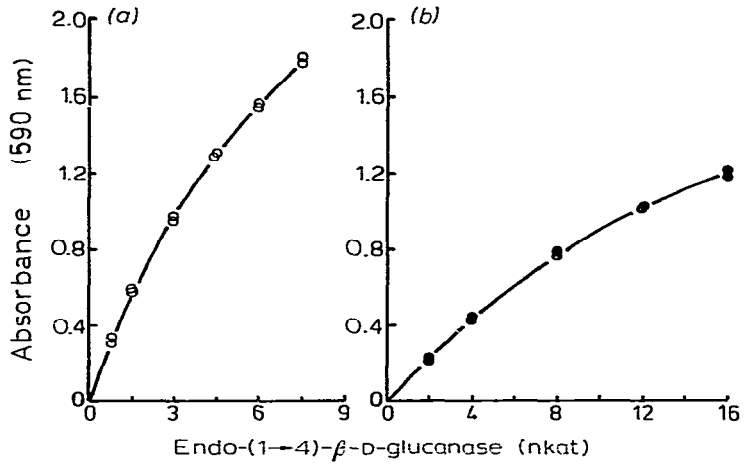


Fig. 1. Standard curves relating nkat of (1→4)-β-D-glucanase activity on CM-cellulose (4M6SF) to absorbance increase (at 590 nm) on hydrolysis of R-Black-CM-cellulose (○) and RBB-CM-cellulose (●) by (1→4)-β-D-glucanase from Driselase preparation. Assay procedures are described in the text and incubations were for 10 min.

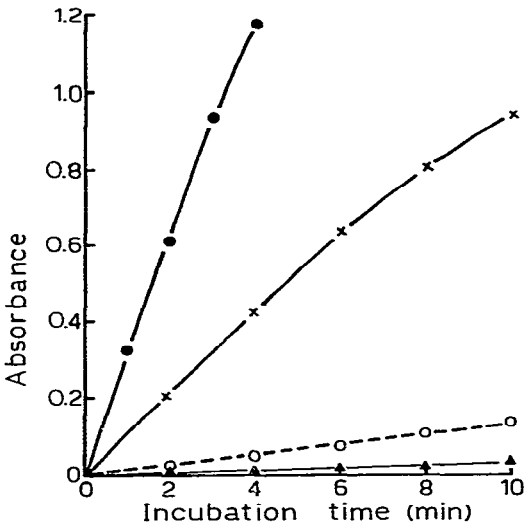


Fig. 2. Relative rates of release of chromogenic material on hydrolysis of R-Black-CM-cellulose (●, 590 nm), RBB-CM-cellulose (×, 590 nm), TNP-cellulose (○, 344 nm), and Cellulose Azure (▲, 590 nm) by (1→4)-β-D-glucanase from Driselase preparation (0.1 ml, 10 nkat). Assay procedures are described in the text.

the degree to which the partially hydrolyzed, dyed substrate precipitated. To ensure effective and reproducible precipitation, high concentrations of buffer salt (0.3M sodium acetate, pH 5) were included in the precipitant solution.

(1→4)-β-D-Glucanase assay. — The two chromogenic substrates used in this assay were obtained by dyeing CM-cellulose (d.s. ~0.4) with Remazolbrilliant Blue

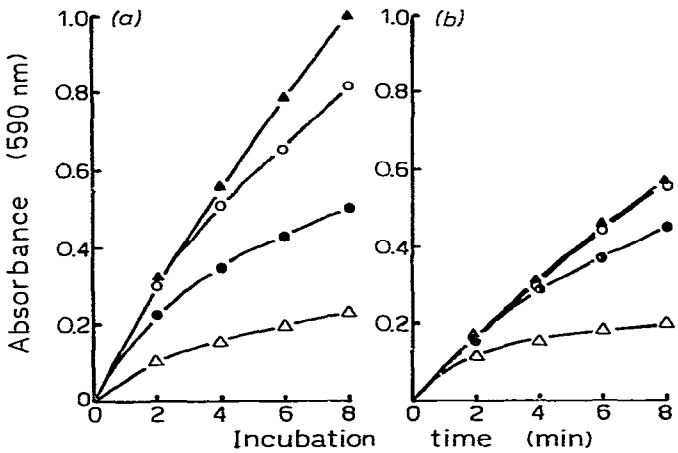


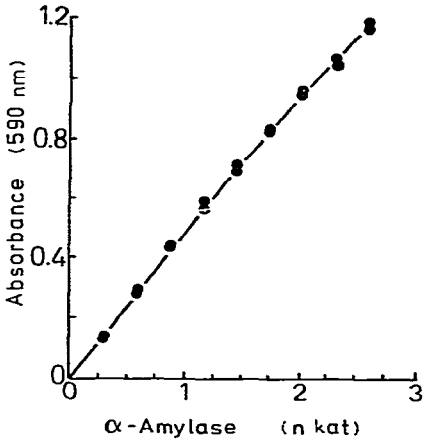
Fig. 3. Effect of substrate concentration on the rate of release of dyed material of low d.p. on hydrolysis by (1→4)-β-D-glucanase of (*a*) R-Black-CM-cellulose (5 nkat of enzyme per assay), and (*b*) RBB-CM-cellulose (7.5 nkat of enzyme per assay). Substrate concentrations: 0.2% (△), 0.5% (●), 1.0% (○), and 2.0% (▲).

R or Remazolbrilliant Black B dyes and are termed RBB-CM-cellulose and R-Black-CM-cellulose. RBB-CM-cellulose was completely soluble in water, whereas R-Black-CM-cellulose gave a fine, gelatinous suspension. Both were readily dispensed accurately and reproducibly. Standard curves for the conversion of absorbance values (at 590 nm) on hydrolysis of these dyed substrates into nkat (at 40° and pH 5) on CM-cellulose (d.s. ~0.4), are shown in Fig. 1. The assay procedure employing R-Black-CM-cellulose was ~3 times more sensitive than that employing RBB-CM-cellulose. The time course of hydrolysis of these two substrates and of TNP-cellulose and Cellulose Azure is shown in Fig. 2. Assays employing R-Black-CM-cellulose were ~100 times more sensitive than those employing Cellulose Azure and 30 times more sensitive than when TNP-cellulose[9] was the substrate.

Both R-Black-CM-cellulose and RBB-CM-cellulose were resistant to hydrolysis by β-D-glucosidase. Incubation of either substrate with β-D-glucosidase (200 nkat, Sigma, G8625) for up to 4 h did not release any dyed fragments soluble in the presence of the precipitant solution.

The effect of substrate concentration on the sensitivity of the assays is shown in Fig. 3. Initial rate curves indicate that the substrates were essentially at saturating concentration at 2% w/v.

Assay of alpha-amylase. — In these studies, chromogenic substrates for the assay of alpha-amylase were prepared by dyeing either CM-amylose or CM-starch with Remazolbrilliant Blue R dye. The CM-d.s. of the amylose was critical in determining both the solubility of the polysaccharide and the susceptibility of the dyed, carboxymethylated polysaccharide to hydrolysis. For amylose, the optimal d.s. to achieve maximal sensitivity was 0.15. However, at this d.s., the polymer, after dyeing, was not completely soluble in buffer solutions. A substrate that was com-



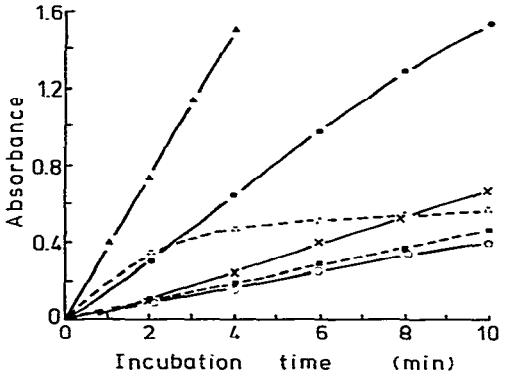


Fig. 4. Relationship between release of chromogenic material of low d.p. from RBB-CM-amylose (d.s. 0.15) and alpha-amylase activity (nkat) on soluble starch. Assay procedures are described in the text. Incubations were for 10 min.

Fig. 5. Rates of release of chromogenic material on hydrolysis of dyed substrates by pancreatic alpha-amylase (0.1 ml, 3.5 nkat). Substrates are Dyamyl-L (▲, 540 nm); RBB-CM-amylose (●, 590 nm); Phadebas (×, 590 nm); Amylopectin Azure (△, 590 nm); Amylochrome (○, 625 nm); and RBB-amylose (■, 590 nm). Assay procedures are described in the text.

pletely soluble in buffer solutions was obtained by increasing the d.s. with CM-groups to ~0.2. However, this d.s. decreased the initial hydrolysis rate of the substrate by almost half, when pancreatic alpha-amylase was used. Dyed CM-soluble starch was completely soluble when the CM-d.s. was only 0.1, but the sensitivity of assays using this substrate was similar to that when RBB-CM-amylose with a CM-d.s. of 0.2 was the substrate.

Although RBB-CM-amylose (d.s. ~0.15) was not completely soluble in buffer solutions, it remained completely suspended for up to 1 h and it could be dispensed accurately and reproducibly. Since it was the most sensitive substrate, it was used for further studies.

A curve relating nkat of alpha-amylase (soluble-starch substrate) to absorbance increase at 590 nm on hydrolysis of RBB-CM-amylose by pancreatic alpha-amylase is shown in Fig. 4. At levels of alpha-amylase above 2 nkat, the substrate became limiting in the assay system described here.

The relative sensitivity of RBB-CM-amylose and other chromogenic substrates in the assay of pancreatic alpha-amylase is shown in Fig. 5. Details of the individual assay procedures are given in Materials and Methods. Assays employing RBB-CM-amylose are approximately twice as sensitive as those employing Phadebas tablets and 3 times more sensitive than those employing Amylochrome tablets. Dyed fragments were rapidly released from Amylopectin Azure, but at 2% substrate concentration, the shape of the initial rate curve and the persistence of insoluble substrate in the assay tube indicated that only a small proportion of the substrate was susceptible to alpha-amylase hydrolysis.

Of the substrates tested, Dyamyl L was the most sensitive. However, a factor limiting its use is its high cost. This is probably due, in part, to the difficulties associated with removing unbound dye from the dyed polymer during its preparation. Since the dyed polymer cannot be readily resuspended after alcohol precipitation, free dye must be removed[3] by gel filtration of the mixture on Sephadex G-25.

Studies of the effect of the concentration of substrate on the rate of release of dyed fragments from RBB-CM-amylose by pancreatic alpha-amylase indicate that a concentration of 2% is sufficient to give maximal sensitivity.

Highly purified preparations of amyloglucosidase or beta-amylase (devoid of alpha-amylase) were unable to release soluble, dyed materials from RBB-CM-amylose, indicating that this substrate is highly specific for alpha-amylase.

The techniques employed here for the preparation of chromogenic substrates of increased solubility and sensitivity for the assay of alpha-amylase and (1→4)-β-D-glucanase also apply to the preparation of soluble, chromogenic substrates for the assay of other polysaccharide endo-hydrolases, *e.g.*, (1→4)-β-D-xylanase. Studies are in progress to assess the application of the alpha-amylase assay procedure, described herein, for the routine determination of alpha-amylase activity in human serum and urine samples and in pre-harvest sprouted, rain-damaged wheat grains.

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