

A long-wavelength fluorescent substrate for continuous fluorometric determination of cellulase activity: resorufin- β -D-cellobioside

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Abstract

A simple and reliable continuous assay procedure for measurement of cellulase activity from several species using the new substrate resorufin- β -D-cellobioside (Res-CB) has been developed. The product of enzyme reaction, resorufin, exhibits fluorescence emission at 585 nm with excitation at 571 nm and has a pK_a of 5.8, which allows continuous measurement of fluorescence turnover at or near physiological pH values. The assay performed using purified cellulase from the microscopic fungus *Trichoderma reesei* has been shown to give the kinetic parameters K_m of 112 μ M and V_{max} of 0.000673 μ mol/mL/min. Methods for performing the assay using cellulases isolated from both live *Arabidopsis thaliana* plant and *Aspergillus niger* fungal species are presented.

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Cellulases are an important family of enzymes found in many plant, bacterial, fungal, and yeast species. The cellulase enzymes [EC 3.2.1.4] hydrolyse the β -1 \rightarrow 4 glucosidic bonds linking glucose units comprising the repeating units of cellulose [1,2]. Cellulases are also divided into several subclasses of isozymes based upon their function: 1,4- β -glucosidases [EC 3.2.1.74], which cleave internal bonds of cellobiose into individual glucose molecules [2], exoglucanases (1,4- β -D-glucan cellobiohydrolase [EC 3.2.1.91]), which cleave cellobiose units from the end of the cellulose chain [1,3], and endoglucanases (1,4- β -D-glucan glucanohydrolase [EC 3.2.1.6]), which cleave the chain at random internal positions [1–3], creating new ends for exoglucanases [3]. Traditionally, cellulolytic enzymes have been defined strictly as belonging to a single class. However, some endoglucanases have been shown to also exhibit exoglucanase activities [4]. Conversely, some exoglucanases may also have endoglucanase functions [3]. Functional specificity can vary among different enzymes, with some enzymes

limited strictly to a single type of cellulolytic activity, whereas others can perform multiple cellulolytic activities to varying degrees [1].

The filamentous fungus *Trichoderma reesei* represents one of the most commonly studied fungal cellulolytic systems [1,6]. The primary component of the organism's cellulolytic system is an exoglucanase, CBHI [1,6], which accounts for over 60% of the total secreted protein produced by *T. reesei* [1,6]. While *T. reesei* is more commonly studied, another filamentous fungus, *Aspergillus niger*, is the most common source of cellulase used commercially [1,5]. Unlike *T. reesei*, the major cellulase of *A. niger* has been found to be an endoglucanase (of Cel12-type) [1,5]. However, *A. niger* also encodes genes for exoglucanases, suggesting that it is a system with multiple cellulolytic functions [1,6]. The cellulases isolated from both *T. reesei* and *A. niger* have found widespread commercial use, namely in the food, detergent, and textile industries. Recent interest in cellulase has increased due to its potential usefulness in the conversion of biomass resources to ethanol or other important by-products [7].

Plants encode cellulases that catalyze the cleavage of the internal 1,4- β -linkages of cellulose comprising their own

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tissue [8]. It has been suggested that plant cellulases are essential for many facets of plant development, including abscission [8,9], fruit softening [8,10], wall loosening [8,11], vascular differentiation [8,12], and symbiosis [8,13].

Synthetic conjugates of the disaccharide subunit of cellulose (cellobiosides) have been employed for detection of cellulase activity. Common cellobiosides include fluorogenic substrates such as 4-methylumbelliferyl β -D-cellobioside (4-MUCB) [14] and chromogenic substrates such as *p*-nitrophenyl- β -D-cellobioside [15] and 5-bromo-4-chloro-3-indolyl- β -D-cellobioside (X-CB)¹ [29]. Exoglucanase and endoglucanase from *T. reesei* have been shown to hydrolyze 4-methylumbelliferyl- β -D-cellobioside into the fluorescent compound 4-methylumbelliferone (4-MU) [14]; exoglucanase, endoglucanase, and β -glucosidase from *T. reesei* have been shown to hydrolyze *p*-nitrophenyl- β -D-cellobioside to yield *p*-nitrophenol (PNP) [15]. These data suggest that phenolic cellobiosides can serve as suitable substrates for cellulases of each subclass. However, chromogenic substrates suffer from low sensitivity and the common fluorogenic substrate 4-methylumbelliferyl- β -D-cellobioside cannot be used for continuous fluorometric measurement of cellulase activity, because of its high pK_a .

Cellulases exhibit optimum activity at low pH (4–6) [14]. Using 4-methylumbelliferyl glycoside substrates does not allow for continuous fluorometric measurement of enzyme activity at these low pH values because the released product, 4-methylumbelliferone, has a pK_a of 7.8 and is therefore substantially nonfluorescent at low pH [14]. In addition, the presence of endogenous fluorescence from proteins and cellular components that have absorption and fluorescence wavelengths similar to those of 4-methylumbelliferone (EX: 380; EM: 454) precludes direct measurement of the low-level cellulase activity present in many complex biological systems. To overcome these limitations, we herein describe a new, long-wavelength fluorescent substrate, resorufin- β -D-cellobioside (Res-CB), which can be used to obtain continuous fluorometric measurement of cellulase activity. This substrate releases the red fluorescent fluorophore resorufin (EX: 571; EM: 585), which has a pK_a of 5.8 [16]. Continuous cellulase activity measurement from samples of purified cellulase from the fungus *T. reesei*, from live cell culture of *A. niger*, and from extracts derived from flowering and leaf tissue of *Arabidopsis thaliana* at pH 6.0 are presented. These results indicate

that the new Res-CB substrate can be used to provide continuous fluorometric measurement and kinetic analysis of cellulase activity from a variety of different biological sources at or near their physiological pH values.

Materials and methods

Chemicals and instruments

Resorufin, sodium salt was obtained from Sigma Chemical Co. (St. Louis, MO). Hydrochloric acid, acetic anhydride, and dry pyridine were obtained from Mallinckrodt Chemicals (Phillipsburg, PA). Hydrobromic acid (33 wt % solution in glacial acetic acid), silver carbonate, Amberlite IRC-50 ion-exchange resin, sodium methoxide (25 wt % solution in methanol), and anhydrous dichloromethane were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sym-collidine was from Acros Organics (Morris Plains, NJ). All-chemicals were used without further purification. ¹H-NMR spectra were obtained using a Varian Inova 300-MHz nuclear magnetic resonance instrument. Microtiter plates used were from Becton–Dickinson Bio-Products Division (BD-Falcon plate No. 351172; 96 well, clear, flat bottom). Fluorescence readings were obtained using a Perkin–Elmer HTS 7000 Plus Bio-Assay Reader and HTSoft Analysis Software.

Synthesis

Resorufin-free acid

Resorufin, sodium salt (3.00 g, 12.75 mmol) was dissolved in ice-water (150 mL) and concentrated hydrochloric acid (6 mL) was added with stirring to pH 2. After stirring at 0 °C (3 h), the red-brown precipitate was filtered and washed with water until the filtrate was neutral. The resulting resorufin-free acid was dried in air and in vacuo overnight to yield a dark red solid (2.64 g, 97%), homogeneous by TLC analysis (9:1 CH₂Cl₂:MeOH, R_f = 0.43).

Resorufin cellobioside, heptaacetate

Under an anhydrous N₂ (g) atmosphere, a mixture of acetobromocellobiose (prepared by acetylation of D-cellobiose with excess acetic anhydride and pyridine [28], followed by treatment with HBr in glacial acetic acid) (2.47 g, 3.53 mmol), anhydrous CH₂Cl₂ (20 mL), anhydrous acetonitrile (20 mL), resorufin (500 mg, 2.35 mmol), dry silver carbonate (484 mg, 1.76 mmol), and sym-collidine (465 μ L) were allowed to stir under anhydrous conditions at room temperature overnight, protected from light. The reaction mixture was filtered through a Celite pad and the grey solids were washed with additional CH₂Cl₂ (30 mL). The clear orange filtrate was washed with water (2 \times 200 mL), saturated aqueous sodium bicarbonate solution (1 \times 100 mL), 1 N HCl/H₂O (1 \times 100 mL), and water (1 \times 100 mL). The resulting organic layer was dried over anhydrous sodium sulfate, filtered, evaporated, and dried in vacuo overnight to yield an orange powder (2.34 g).

¹ Abbreviations used: Res-CB, resorufin- β -D-cellobioside; pK_a , acid dissociation constant; CBH1, cellobiohydrolase I; V_{max} , maximum velocity; EX, excitation; EM, emission; 4-MUCB, 4-methylumbelliferyl β -D-cellobioside; PNP, *p*-nitrophenol; CH₂Cl₂, dichloromethane; MeOH, methanol; R_f , retention factor; TLC, thin-layer chromatography; HCl, hydrochloric acid; CDCl₃, deuteriochloroform; Ac, acetate; HOAc, acetic acid; SiO₂, silica; EtOAc, ethylacetate; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; NAD, nicotinamide adenine dinucleotide; 4-MU, 4-methylumbelliferone; Tris, tris-hydroxymethylaminomethane; HPLC, high-pressure liquid chromatography.

Purification by column chromatography (SiO₂, 70–230 mesh, 25 × 75 mm, irrigant = 9:1 CH₂Cl₂:EtOAc) produced a bright orange solid (1.89 g, 97%). (R_f = 0.47 irrigant = 9:1 CH₂Cl₂:MeOH). ¹H-NMR (CDCl₃, 300 MHz): δ 7.80 (d, 1H), 7.56 (d, 1H), 7.10 (dd, 2H), 6.81 (d, 1H), 6.30 (d, 1H), 5.66 (d, 1H), 5.3 (m, 2H), 5.06 (d, 2H), 4.90 (dd, 2H), 4.70 (dd, 2H), 4.40 (d, 1H), 4.28 (m, 2H), 4.0 (m, 3H), 3.7 (m, 3H), 3.2 (m, 2H), 3.10 (m, 2H), 2.1 (s, 3H), 2.0 (s, 6H), 1.98 (s, 6H), 1.95 (s, 3H), 1.92 (s, 3H).

Resorufin-β-D-cellobioside

Resorufin cellobioside, heptaacetate (417 mg, 0.50 mmol) was suspended in anhydrous methanol (15 mL) and sodium methoxide, 25 wt % solution in methanol (300 μL) was added (to pH 10). This reaction was stirred overnight at room temperature, and the resulting bright orange product was filtered, washed with minimum anhydrous methanol, and dried in vacuo overnight to produce an orange solid that was recrystallized from acetonitrile. Yield = 268 mg, 99% TLC (R_f = 0.83, irrigant = 7:3 EtOAc:MeOH). ¹H-NMR (DMSO-d₆, 300 MHz): δ = 7.80 (d, J = 7.8 Hz, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.17 (d, J = 0.8 Hz, 1H), 7.10 (dd, J = 7.8, 0.8 Hz, 1H), 6.79 (dd, J = 7.8, 0.84 Hz, 1H), 6.39 (d, J = 1.3 Hz, 1H), 5.61 (d, J = 6.5 Hz, 1H), 5.21 (dd, J = 13.7, 5.9 Hz, 2H), 5.02 (dd, J = 9.1, 5.9 Hz, 2H), 4.83 (d, J = 0.1 Hz, 1H), 4.65 (m, J = 7.8 Hz, 2H), 4.28 (d, J = 0.7 Hz, 1H), 3.63 (m, 4H), 3.45 (m, 3H), 3.10 (m, 6H).

Growth of *Arabidopsis thaliana*

A. thaliana (strain CS-20) were grown under sterile conditions in Murashige and Skoog media at room temperature using a 16-h daylight photoperiod. The growth chamber utilized a combination of two Philips Plant/Aquarium T12 40-W bulbs and two Philips ALTO Collection T8 32-W bulbs for illumination at a height of 18 inches above seedlings. Plants were grown until floral initiation was evident (approx. 6 weeks) prior to analysis.

Culture of *Aspergillus niger*

Sterile growth medium (50 mL) [17] was inoculated with a 1-mL suspension of *A. niger* (ATCC No. 6275) seed culture, prepared as described previously [17]. Cultures were incubated at room temperature in sterile, loosely capped 125-mL culture flasks, with stirring on an orbital shaker (120 rpm) until mycelia were evident (7 days) prior to harvesting for analysis.

Assay of purified cellulase

Four dilutions of cellulase isolated from *T. reesei* (1.0, 0.50, 0.10, 0.05 U/mL) (Fluka No. 22173) were prepared in 25 mM sodium acetate buffer, pH 6.0. Each enzyme preparation was added in triplicate (50 μL) to wells in a clear, flat-bottomed 96-well polystyrene microtiter plate

(BD Falcon No. 351172). A blank sample containing no enzyme (50 μL reaction buffer) was also prepared in triplicate wells. A 0.5 mM substrate reagent solution was prepared by diluting a 5 mM DMSO stock 1:10 in 25 mM sodium acetate buffer, pH 6.0. The substrate reagent solution was added to each well to a final concentration of 0.25 mM (50 μL). Fluorescence was measured using a Perkin-Elmer HTS 7000 Plus BioAssay Reader in top read mode (excitation filter = 550 nm, emission filter = 595 nm). Fluorescence was recorded at room temperature for 30 cycles with a cycle time of 1 min. All readings were performed in triplicate and averaged.

Cellulase assay of *Arabidopsis thaliana* tissue samples

Flowering tissue from two mature plants were collected (90 mg total tissue) and ground in liquid nitrogen (30 mL). The resulting powder was suspended in 200 μL 25 mM sodium acetate buffer, pH 6.0. The suspension was centrifuged at 13,000 rpm (17,900g) for 10 min (Eppendorf 5417 C microcentrifuge) [18]. The supernatant was collected and added in triplicate (50 μL) to wells in a clear, flat-bottomed 96-well polystyrene microtiter plate (BD Falcon No. 351172). A blank sample containing no enzyme (50 μL reaction buffer) was also prepared in triplicate wells. A 0.5 mM substrate reagent was prepared by diluting 5 mM DMSO stock 1:10 in 25 mM sodium acetate buffer, pH 6.0, and was added to each well to a final concentration of 0.25 mM (50 μL). Fluorescence was measured using the parameters listed above. Fluorescence was recorded at room temperature for 40 cycles with a cycle time of 3 min. All readings were performed in triplicate and averaged.

Cellulase assay of *Aspergillus niger* exudate

A 1-mL aliquot was removed from a 7-day culture and centrifuged at 13,000 rpm (17,900g) for 10 min (Eppendorf 5417 C microcentrifuge) to remove cell debris. The supernatant was collected and diluted 1:1 in 100 mM sodium acetate buffer, pH 6.0. Samples were then added in triplicate (50 μL) to wells in a clear, flat-bottomed 96-well polystyrene microtiter plate (BD Falcon No. 351172). A blank sample containing no enzyme (50 μL reaction buffer) was also prepared in triplicate wells. A 0.5 mM substrate reagent was prepared by diluting 5 mM DMSO stock 1:10 in 100 mM sodium acetate buffer, pH 6.0. This substrate reagent was added to each well to a final concentration of 0.25 mM (50 μL) and fluorescence measured as above. Fluorescence was recorded at room temperature for 20 cycles with a cycle time of 3 min. All readings were performed in triplicate and averaged. Immediately following the final cycle, the reaction was terminated by addition of 165 mM Tris buffer, pH 10.0 (30 μL). Resorufin was added to triplicate wells to a final concentration of 0.25 mM (50 μL) in sodium acetate buffer (50 μL) for use as a reference standard. Tris buffer was also added to

blanks and reference standards, and an endpoint measurement was recorded using the above parameters.

Continuous kinetic assay

Five Res-CB substrate reagent solutions (0.50, 0.40, 0.30, 0.20, 0.10 mM) were prepared by diluting DMSO stocks 1:10 in 25 mM sodium acetate buffer, pH 6.0. The substrate reagent solutions were added in triplicate (50 μ L) to wells in a clear, flat-bottomed 96-well polystyrene microtiter plate (BD Falcon No. 351172). Cellulase isolated from *T. reesei* (0.10 U/mL, Fluka No. 22173) was prepared in 25 mM sodium acetate buffer, pH 6.0, and added to the above wells (50 μ L). Final substrate concentrations were 0.25, 0.20, 0.15, 0.10, and 0.050 mM. Fluorescence was measured immediately using a Perkin–Elmer HTS 7000 Plus BioAssay Reader in top read mode (excitation filter = 550 nm, emission filter = 595 nm). Fluorescence was recorded for four 30-s cycles, and the mean fluorescence value of a blank (50 μ L substrate reagent added to 50 μ L reaction buffer, triplicate wells) was subtracted from the value of each sample well to normalize data at each time point. A standard curve was generated by plotting fluorescence of four concentrations of resorufin standards (50, 20, 10, 5 μ M) prepared by diluting DMSO stocks in sodium acetate buffer and adding to microtiter plate wells in triplicate (100 μ L). All readings from triplicate wells were averaged. The curve generated from the standards was used to convert raw fluorescence data into μ mol mL⁻¹ min⁻¹ resorufin produced. Initial velocities were determined from the linear portion of this curve using Graft and Microsoft Excel graphing software.

Kinetic assay with stop buffer

Five Res-CB substrate reagent solutions (0.50, 0.40, 0.30, 0.20, 0.10 mM) were prepared by diluting DMSO stocks 1:10 in 25 mM sodium acetate buffer, pH 6.0. The substrate reagent solutions were added in triplicate (50 μ L) to wells in a clear, flat-bottomed 96-well polystyrene microtiter plate (BD Falcon No. 351172). Cellulase isolated from *T. reesei* (0.10 U/mL, Fluka No. 22173) was prepared in 25 mM sodium acetate buffer, pH 6.0, and added to wells (50 μ L). Final substrate concentrations were 0.25, 0.20, 0.15, 0.10, and 0.05 mM. The assay was performed four times, with 165 mM Tris buffer, pH 10.0 (stop buffer) (30 μ L) being added to wells at $t = 30, 60, 90,$ and 120 s. Fluorescence was measured using a Perkin–Elmer HTS 7000 Plus BioAssay Reader in top read mode (excitation filter = 550 nm, emission filter = 595 nm). The mean fluorescence value of a blank (50 μ L substrate reagent added to 50 μ L reaction buffer, followed by addition of 30 μ L stop buffer, triplicate wells) was subtracted from the value of each sample well to normalize data at each time point. A standard curve was created by plotting fluorescence of four known

concentrations of resorufin standards (50, 20, 10, 5 μ M), prepared by diluting DMSO stocks in sodium acetate buffer and adding to microtiter plate wells in triplicate (100 μ L), followed by 30 μ L stop buffer. Readings in triplicate wells were averaged. This plot was used to convert fluorescence data into μ mol/mL/min resorufin produced. Initial velocities were determined from the linear portion of this curve using Graft and Microsoft Excel graphing software.

Results and discussion

Several fluorescent and chromogenic cellobiosides are commercially available for detection of cellulase activity. However, kinetic assays using these substrates for continuous assay must be obtained either by photometric analysis of product turnover using cumbersome thin-layer chromatography or by HPLC analysis of aliquots removed at various time points, even when using the common fluorogenic substrate 4-methylumbelliferyl- β -D-cellobioside [14]. The enzymatic product, 4-MU has a pK_a value of 7.8 while most cellulolytic enzymes have optimal activity in a pH range of 4.0–6.0. 4-MU fluorescence is, therefore, minimal when performing assays at an acidic pH. Nevertheless, fluorometric data have been obtained in the form of endpoint readings, by removing aliquots at various times during the enzyme reaction and adding a high-pH buffer to elevate the pH of the reaction mixture above the pK_a of 4-methylumbelliferone [14] (i.e., adding a “stop buffer”). But this method is time-consuming and limits the ability to perform sensitive kinetic analyses of the enzymatic reaction at early time points. Other chromogenic substrates, including 2-chloro-4-nitrophenyl- β -D-cellobioside, 2,4-dinitrophenyl- β -D-cellobioside, 3,4-dinitrophenyl- β -D-cellobioside, 2,5-dinitrophenyl- β -D-cellobioside [19], or 4-nitrophenyl- β -D-cellobioside require similar methods for analysis, and 5-bromo-4-chloro-3-indoxyl-*N*-acetyl- β -D-cellobioside (X-cellobioside) generates a dark-blue precipitate that is difficult to quantitate.

We reasoned that an alternate fluorophore might aid continuous cellulase measurements. Resorufin is a long-wavelength, red-fluorescent fluorophore (EX: 571, EM: 585) with a pK_a of ~ 5.8 [16]. Its low pK_a relative to that of 4-methylumbelliferone presented resorufin- β -D-cellobioside as a superior candidate for continuous fluorometric determination of cellulase activity because its product from cellulase reaction, resorufin, should retain appreciable fluorescence at low pH values. The use of resorufin-based substrates should therefore make it possible to obtain continuous fluorometric measurements and kinetic enzyme data for a variety of cellulases at or near their physiological pH values. In addition, the longer-wavelength excitation and emission values for resorufin might also allow improved measurement in the presence of endogenous blue and green fluorescent species found in many plant, fungal, or bacterial tissues [16,21].

Synthesis of resorufin β -D-cellobioside

Our synthetic methods were patterned on those used for other similar resorufin glycosides [20], employing resorufin, free phenol as starting material, prepared by acid treatment and crystallization of commercially available resorufin, sodium salt. The glycosylation reaction utilized the peracetylated 1-deoxy-1-bromo derivative of cellobiose that could be prepared by sequential acetylation and treatment of the anomeric mixture of α - and β -cellobiose, octaacetates with HBr in glacial acetic acid at 0 °C. The temperature during synthesis of the bromo-sugar was maintained at 0 °C throughout the reaction because it was found that allowing the temperature to warm, even to room temperature, caused partial hydrolysis of cellobiose into glucose. Using these methods, high yields of the reactive bromo-sugar product were produced, absent of free glucose. The anomeric configuration of the acetobromocellobiose produced was found to be exclusively α , as evidenced by the $^1\text{H-NMR}$ analysis.

Glycosylation (Fig. 1) was carried out utilizing a modified Koenigs–Knorr methodology [26] employing silver carbonate as catalyst and sym-collidine as proton scavenger to give the protected intermediate resorufin- β -D-cellobioside, heptaacetate. Zemplen deprotection [27] with catalytic sodium methoxide in methanol afforded the desired Res-CB substrate as a single product that crystallized from the reaction mixture in virtually quantitative yield.

The Res-CB substrate thus produced was found to be stable and could be stored for long periods of time at $-18\text{ }^\circ\text{C}$ (6 months), desiccated in a powdered form or in

anhydrous DMSO solution (5 mM), without noticeable decomposition.

Enzymatic assays from tissue culture samples

Our results demonstrate the ability of this new substrate to provide continuous fluorometric measurement of cellulase activity from several different sources. At pH 6.0, increased fluorescence over time was observed in assays of purified cellulase from *T. reesei* (Fig. 2), from extracts of flowering tissue of *A. thaliana* (Fig. 3), and from the liquid culture supernatant of *A. niger* (Fig. 4).

A lag phase of several minutes of the enzyme reaction in both the *Aspergillus* and the *Arabidopsis* extracts was observed before steady state kinetics was initiated. Such lag phases are known and have been attributed to a variety of phenomena, including changes in the aggregation state of the enzyme, conversion of the enzyme from inactive to active forms through accumulation of cofactors [22], auto-catalytic activity of the enzyme [23], or by the presence of an inhibitory compound that must be displaced before the reaction can proceed [24]. The lag phase observed was similar to that described for the NAD malic enzyme isolated from plant tissue (*Crassula argentea*) which has been demonstrated to be longest just after sample preparation and varies depending on buffer or concentrations of the enzyme or substrate [22].

In the assay of cellulases derived from *A. niger*, a 100 mM sodium acetate buffer was used (in place of the 25 mM buffer used in assays of *Trichoderma* and *Arabidopsis* cellulases) to sufficiently buffer the particularly acidic pH of

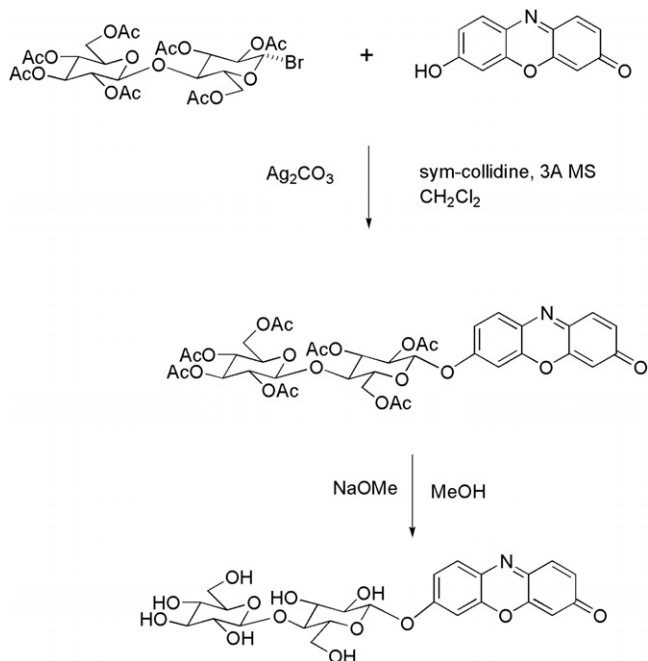


Fig. 1. Scheme for synthesis of resorufin- β -D-cellobioside.

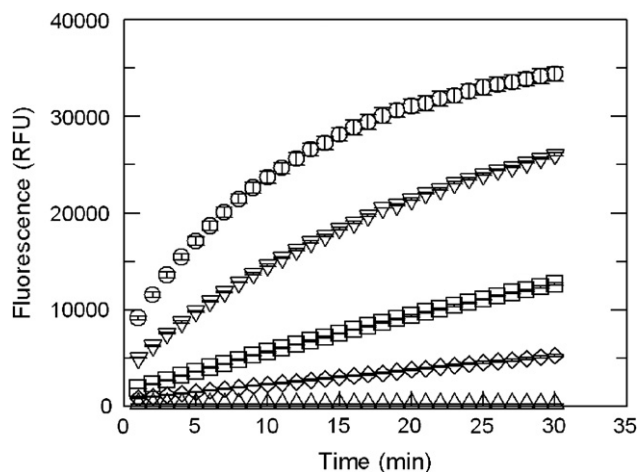


Fig. 2. Continuous fluorescent analysis of purified cellulase from *Trichoderma reesei* (Fluka Prod. No. 22173) using the substrate resorufin- β -D-cellobioside (Res-CB). Enzyme samples (1.0 U/mL (\circ), 0.50 U/mL (∇), 0.10 U/mL (\square), 0.05 U/mL (\diamond)) were prepared in 25 mM sodium acetate buffer, pH 6.0. Res-CB (50 μL) was added to the enzyme samples (50 μL) to a final concentration of 0.25 mM. Fluorescence of a blank (no-enzyme) sample (\triangle) was also analyzed over the same time period. Fluorescence emission was measured at 590 nm with excitation at 550 nm for 30 min at 1-min intervals. Data points are means, and error bars represent standard errors ($n = 3$).

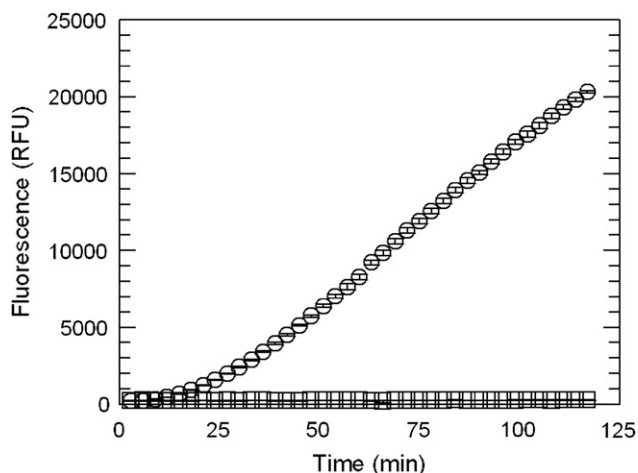


Fig. 3. Continuous fluorescent analysis of cellulase from an extract of flowering tissue of *Arabidopsis thaliana* Landsberg Erecta plants (○). Extracts were prepared by grinding plant tissue (90 mg) in liquid N₂ (30 mL) and suspending the resulting solid in 25 mM sodium acetate buffer, pH 6.0 (200 μL), followed by centrifugation (17,900g) to remove debris. Res-CB (50 μL) was added to the extract solution (50 μL) to a final concentration of 0.25 mM. Fluorescence of a blank (no-enzyme) sample (□) was also analyzed over the same time period. Fluorescence emission was measured at 590 nm with excitation at 550 nm every 3 min for a total of 120 min. Data points are means, and error bars represent standard errors ($n = 3$).

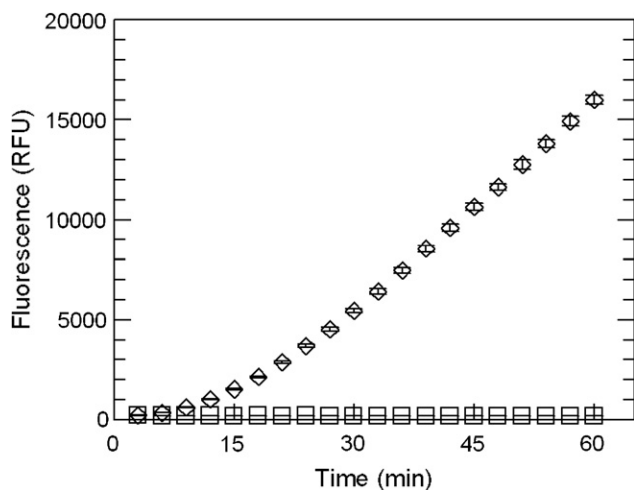


Fig. 4. Continuous fluorescent analysis of cellulase from culture exudate of *Aspergillus niger* (◇). An aliquot (1 mL) from a healthy exponentially growing liquid culture (7 days after inoculation) was removed and centrifuged (17,900g) to remove cells and debris. A portion of the supernatant was diluted 1:1 in 100 mM sodium acetate buffer, pH 6.0 (final volume 50 μL), and Res-CB was added at a final concentration of 0.25 mM (50 μL). Fluorescence of a blank (no-enzyme) sample (□) was also analyzed over the same time period. Fluorescence emission was measured at 590 nm with excitation at 550 nm every 3 min for a total of 60 min. Data points are means, and error bars represent standard errors ($n = 3$).

the *Aspergillus* culture supernatant samples. We suggest that high buffer capacity be employed for continuous fluorometric measurements of other strongly acidic samples.

In previous studies, the substrate 4-MUCB has been used for determination of cellulase activity, by measuring either the UV absorbance changes upon enzyme activity or the fluorescence endpoint at various time points with the addition of a high pH stop buffer to terminate the reaction and increase the fluorescence of the released fluorophore [14]. In the latter method, aliquots are removed after various incubation periods and treated with the stop buffer to raise the pH and develop the fluorescence prior to analysis. This method has been used to determine the cellulase activity of low-pH samples, albeit not in a continuous format (Fig. 5). In addition, the products from these reactions (4-MU, glucose) have been measured by cumbersome TLC or HPLC analysis techniques. Nevertheless, using this method, the K_m and V_{max} values for cellulase activity toward 4-MUCB by *T. reesei* endo-1,4-β-glucanase have been reported to be $K_m = 1.2 \pm 0.1$ mM with a maximum velocity $V_{max} = 9$ μmol/min/mg purified enzyme [14].

In a similar manner, we repeated this stop buffer method for measurement of the fluorescence obtained using our new Res-CB substrate by the addition of a Tris stop buffer (to pH 10.0) and compared the results to those obtained using the continuous assay format. This stop buffer method elevates the pH and increases the fluorescence emission (Fig. 5) of the released resorufin product relative to its fluorescence at pH 6.0 (Fig. 4). However, we found virtually no change in the relative kinetics in the two methods. Using the stop buffer method, the kinetic assay parameters for the Res-CB assay were found to be comparable to those obtained in a continuous assay format (Fig. 6). The kinetic parameters for the Res-CB substrate with purified enzyme from *T. reesei* using the stop buffer technique (addition of

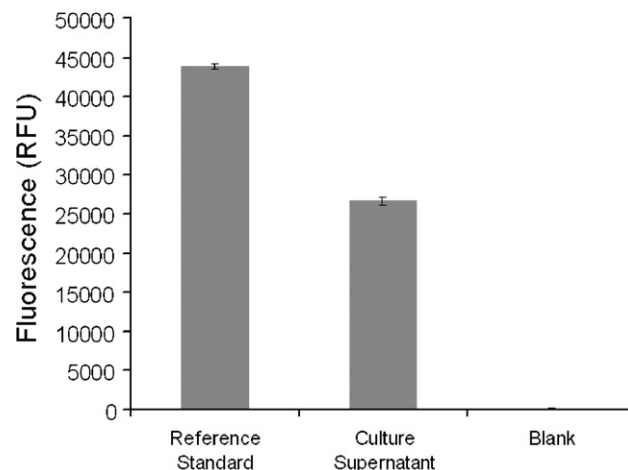


Fig. 5. Comparison of fluorescence readings for reference standard (0.25 mM resorufin in sodium acetate buffer), Res-CB (0.25 mM final concentration) added to culture supernatant of *Aspergillus niger* in sodium acetate buffer, 60 min incubation), and blank (0.25 mM Res-CB in sodium acetate buffer, no enzyme, 60 min incubation) followed by addition of a stop buffer (30 μL) (165 mM Tris, pH 10.0) to each sample to elevate the pH to 9.5 and terminate the reaction. Total volume in wells prior to addition of stop buffer was 100 μL. Data points are means, and error bars represent standard errors ($n = 3$).

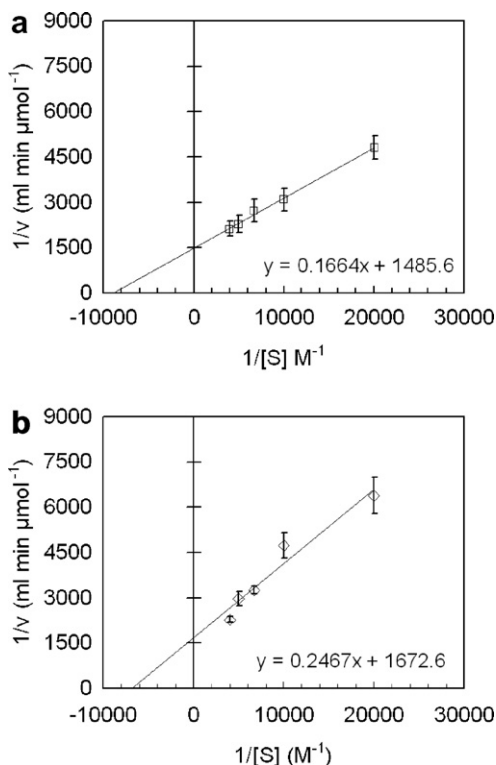


Fig. 6. Lineweaver–Burk Plots generated from the continuous and stop buffer kinetic assay methods: (a) Continuous assay format; (b) assay method utilizing addition of a stop buffer (pH 10.0) at several time points. Experimentally determined values from continuous assay (a) for K_m and V_{max} were 112 μM and 0.000673 $\mu\text{mol}/\text{mL}/\text{min}$, respectively. Values for K_m and V_{max} determined by stop buffer assay method (b) were 147 μM and 0.000598 $\mu\text{mol}/\text{mL}/\text{min}$, respectively. Continuous assay method (a) was performed by adding purified cellulase (50 μL) from *Trichoderma reesei* (Fluka 22173) (0.1U/mL) to five concentrations (0.25, 0.2, 0.15, 0.10, 0.05 mM) of Res-CB in 25 mM sodium acetate buffer, pH 6.0 (50 μL). Fluorescence was recorded (EX: 550, EM: 595) at 30-s time intervals for 120 s. Data were converted to μmol of resorufin product produced using a standard curve created using known concentrations of resorufin in sodium acetate buffer (pH 6.0). Stop buffer assay method (b) was performed using four simultaneous assays using purified cellulase from *T. reesei* (Fluka 22173) (0.1 U/mL) added to five concentrations (0.25, 0.2, 0.15, 0.10, 0.05mM) of Res-CB (as in (a)) in 25 mM sodium acetate buffer, pH 6.0. A stop buffer (165 mM Tris, pH 10.0) (30 μL) was added to assays at 30, 60, 90, and 120 s to elevate the pH to 9.5 and terminate the reaction. Fluorescence of all assays was recorded using EX: 550; EM: 595. Data were converted to μmol of resorufin product produced using a standard curve created by adding stop buffer to known concentrations of resorufin in sodium acetate buffer (pH 6.0). Data points are means, and error bars represent standard errors ($n = 3$).

Tris buffer, pH 10.0, to terminate the reaction at several time points) produced a measured K_m of 147 μM and a V_{max} of 0.000598 $\mu\text{mol}/\text{mL}/\text{min}$ (Fig. 6b). The continuous assay, performed using a pH 6.0 buffer throughout, which is closer to the physiological pH found in most native species, exhibited corresponding kinetic data of K_m of 112 μM and a V_{max} of 0.000673 $\mu\text{mol}/\text{mL}/\text{min}$ (Fig. 6a). Because cellulases are inactive at high pH values, it is impossible to measure their activity at high pH values, but these data suggest that the continuous assay performed at pH 6.0 with the new Res-CB can be used to obtain reliable kinetic data

at or near physiological pH values. Although fluorescence emission of the released resorufin product at pH 6.0 is somewhat reduced (approximately 30%) relative to its fluorescence at a higher pH, the kinetic parameters obtained remained essentially unaffected. We also found the Res-CB substrate to be stable under high-pH conditions, exhibiting very low background (nonspecific) turnover (no-enzyme blank) for up to 12 h at pH 9.0. Furthermore, since resorufin is a relatively pH-insensitive fluorophore, fluorescence emission and excitation wavelengths remain fairly uniform over a large range of pH values from 3–9 [25] which will allow the same filter sets and instrumental setup methods to be used for assays run with different samples having a variety of pH values. It is noted that fluorescence measurements of free resorufin standards should be measured over the range of concentrations and at the same pH as those used for test samples to obtain quantitative turnover rates at specific pHs.

The enzyme specificity of the new Res-CB substrate toward the various cellulase isozymes has not been determined, but we anticipate that it will most likely pattern those of other phenolic aglycone substrates [14], showing activity for β -glucosidases, exoglucanases, and some endoglucanases (such as *T. reesei* endoglucanase II) but low or no activity with other endoglucanase enzymes (such as *T. reesei* endoglucanase III and *Methanoseta thermophila* endoglucanase) [14]. Work is underway to measure the enzyme specificities toward additional cellulase isozymes of Res-CB and compare these data to those of both 4-MU- and PNP-based cellulase substrates.

Uses of the new Res-CB substrate for continuous assay of cellulases derived from additional species and in live cell or live tissue formats are underway and should facilitate the analysis of cellulase activity in a variety of important biological and bioengineering systems.

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