A Bifunctionalized Fluorogenic Tetrasaccharide as a Substrate to Study Cellulases*

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Cellulases are usually classified as endoglucanases and cellobiohydrolases, but the heterogeneity of cellulose, in terms of particle size and crystallinity, has always represented a problem for the biochemical characterization of the enzymes. The synthesis of a bifunctionalized tetrasaccharide substrate suitable for measuring cellulase activity by resonance energy transfer is described. The substrate, which carries a 5-(2- aminoethylamino)-1-naphthalenesulfonate group on the non-reducing end and an indolethyl group on the reducing end, was prepared from β-lactosyl fluoride and indolethyl β-celllobioside by a chemoenzymatic approach using the transglycosylating activity of endoglucanase I of Humicola insolens as the step. The bifunctionalized substrate has been used for the determination of the catalytic constants of H. insolens endoglucanase I and cellobiohydrolases I and II; this substrate could be of general use to measure the kinetic constants of cellulases able to act on oligomers of degree of polymerization <5. The data also provide evidence that cellobiohydrolases I and II are able to degrade an oligosaccharide substrate carrying non-carbohydrate substituents at both ends.

Because of the abundance, the properties, and the many uses of cellulose, cellulolytic enzymes have considerable ecological as well as industrial importance. However, the heterogeneity of native cellulose, in terms of particle size and crystallinity, has always represented a problem for the biochemical characterization of cellulases. Small soluble substrates containing a chromophoric group at the reducing end have proved to be very useful for the specificity mapping of various cellulases (6). However, the use of these substrates for kinetic studies is limited since the enzymatic cleavage is monitored directly only when it occurs between the sugar and the aglycon.

The aim of this work is the synthesis of a bifunctionalized tetrasaccharide substrate for cellulases to provide a sensitive assay to determine the kinetic parameters of different cellulases using micromolar concentrations of substrate. This substrate contains two fluorogenic groups, respectively, at the reducing and non-reducing end of the molecule. The principle of the assay is based on the fact that the chromophore donor and the fluorophore acceptor are in sufficient proximity in the substrate to allow a resonance energy transfer between the two fluorogenic groups. After hydrolysis of any glycosidic bond in the substrate, the two fluorogenic groups are separated, and the resonance energy transfer is no longer possible. The decrease in resonance energy transfer therefore provides a convenient way to monitor the enzymatic reaction. This strategy has been used previously for the assay of proteolytic enzymes (7, 8) and of α-amylases (9). To overcome the problems related to a chemical synthesis of a complex tetrasaccharide containing four β-1,4-linkages, a chemoenzymatic approach has been developed for the preparation of the bifunctionalized substrate. A similar strategy was used recently for obtaining a disubstituted maltopentaoside for the assay of α-amylases (9). The enzyme used for the enzymatic condensation is the recombinant endoglucanase I from the fungus Humicola insolens, which has previously been shown to exhibit a high transglycosylating activity (10).

The preparation of the tetrasaccharide indolethyl 6-N-6-(5-(2-aminoethylamino)-1-naphthalenesulfonate)-6-deoxy-β-D-galactopyranosyl-4-O-(β-D-glucopyranosyl)-4-O-β-D-glucopyranoside IX, is reported (Fig. 1). This substrate, which provides a sensitive assay for cellulolytic enzymes, proved useful for the determination of the kinetic parameters of several recombinant cellulases from H. insolens.

EXPERIMENTAL PROCEDURES

Enzymes

The H. insolens cellulases were all cloned and expressed in Aspergillus oryzae (11–13). Endoglucanase V (EGV) and cellobiohydrolase I (CBHI) were purified using Avicel affinity chromatography as described (14). EGV and CBHI gave single bands in SDS-polyacrylamide gel electrophoresis at 43 and 70 kDa, respectively. Their respective molar absorption coefficients were of 61,300 and 89,700 cm⁻¹ m⁻¹. Endoglucanase I (EGI) and cellobiohydrolase II core (CBHII core) were purified...
using ion exchange chromatography. EGI was applied to cation exchange chromatography using Mono S (Pharmacia Biotech.) and 50 mM sodium acetate buffer, pH 5.0. Pure EGI was eluted using a pH gradient from 50 mM sodium acetate buffer, pH 5.0, to 100 mM potassium phosphate, pH 7.0. EGI showed a single band in SDS-polyacrylamide gel electrophoresis (150 kDa) at a molar absorption coefficient of 66,300 cm⁻¹ m⁻¹. CBHII core was purified by anion exchange chromatography (DEAE-Sepharose) and 20 mM triethanolamine buffer, pH 8.0. CBHII core was eluted using a 0–1 M sodium chloride gradient.

The enzyme was purified further by size chromatography on S-300 Sephacyr (Pharmacia) using 0.1 M sodium acetate buffer, pH 6.1. Purified CBHII core gave a single band in SDS-polyacrylamide gel electrophoresis at 42 kDa and had a molar absorption coefficient of 77,260 cm⁻¹ m⁻¹.

Methods

All of the new compounds displayed elemental analysis and NMR and mass spectra in accordance to their structure. NMR spectra were recorded on a Bruker AC 300 spectrometer at 300 MHz for 1H and 75 MHz for 13C and on a Varian Unity Plus at 500 MHz for 1H. The mass spectra were recorded on a Nermag R-1010C spectrometer. Optical rotations were measured at 20 °C on a Perkin-Elmer 241 polarimeter. The enzymatic condensation reaction was analyzed by HPLC using an analytical NH2 column (BONDAPAK, Waters) equipped with a refractometer and eluted with acetonitrile.

Preparation of β-Lactosyl Fluoride (II)—Hepta-α-acetylactosyl bromide I was prepared in a conventional fashion (15). β-Lactosyl fluoride II was synthesized by reacting hepta-α-acetylactosyl bromide I (14 g; 0.02 mol) with silver fluoride (5.2 g; 0.04 mol) in anhydrous acetonitrile (156 ml) for 4 h at 22 °C. The hepta-α-acetylactosyl fluoride product was purified by flash chromatography on silica gel (ethyl acetate/light petroleum, 1:1) in quantitative yield. Reductive amination of bromide I with 3-(2-hydroxyethyl)indole (156 ml) for 4 h at 2 °C. The hepta-α-acetylactosyl fluoride II was neutralized with an H²⁺ resin, recovered, and used immediately without further characterization.

Preparation of Indolethyl β-Cellobioside (V)—Hepta-α-acetylcellobiosyl bromide III was prepared in a conventional fashion (15). Glycosylation of bromide III (2.9 g, 4.15 mmol) with 3-(2-hydroxyethyl)indole (33 g; 0.23 mmol) was achieved in anhydrous toluene (45 ml) and nitromethane (40 ml) in the presence of Hg(CN)₂ (950 mg) and HgBr₂ (67 mg), for 18 h at 22 °C. The expected disaccharide IV was purified by flash chromatography on silica gel (ethyl acetate/light petroleum, 1:1 v/v), isolated with a yield of 40% and characterized: mp 78–80 °C; 1H NMR (300 MHz, CDCl₃) δ = 5.32 (dd, J1H,1F, 5 Hz), F = 53 Hz, J = 1H,2 = 5 Hz, H,1; 13C NMR (75.4 MHz, CDCl₃) δ = 170.16–169.00 (7 × CO), 105.55 (d, J1C,1F, 2 = 218 Hz, C,1), 101.12 (C,2), 20.88–20.34 (7 × CH₂). After deacetylation in the presence of 0.5 M sodium methoxide in methanol for 3 h at 0 °C, β-lactosyl fluoride II was neutralized with an H⁺ resin, recovered, and used immediately without further characterization.

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Analysis of the enzymatic hydrolysis of bifunctionalized substrate IX were performed at 37 °C in 0.1 M sodium acetate buffer, pH 6.1. Initial rates of the enzymatic hydrolyses of bifunctionalized substrates were monitored with a Perkin-Elmer LS50 spectrofluorometer using an excitation lambda at 250 nm and an emission lambda at 400 nm.

RESULTS AND DISCUSSION

Chemoenzymatic Synthesis of the Substrate—During the hydrolysis of oligosaccharides and glycosides under thermodynamically controlled conditions, certain retaining glycosyl hydrolases such as β-galactosidase (18), β-glucosidase (17), and β-xylosidase (18) display transglycosylating activity and produce mixtures of higher oligosaccharides in moderate yields and low linkage specificity. Under kinetically controlled conditions, more efficient transglycosylating reactions can be achieved when an activated carbohydrate donor is incubated with a polysaccharidase. In this case, the resulting higher oligosaccharides also exhibit good linkage specificity. This approach was first used for the high yield preparation of maltoligosaccharides using maltotetraosyl fluoride and α-amylase (19).

This strategy has since been used for the preparation of natural or non-natural oligosaccharides using, for instance, amylases (9) or cellulases (20, 21).

Fluorometric Assays and Determination of the Kinetic Constants—The initial rates of the enzymatic hydrolysis of bifunctionalized substrate IX were determined using Lineweaver-Burk plots and were the result of three determinations. All kinetic studies were performed in a Perkin-Elmer LS50 spectrophotometer using an excitation wavelength set at 290 nm and an emission wavelength set at 490 nm.
The principle of the transglycosylation reaction is as follows. The enzyme initially reacts with the only substrate available, i.e. lactosyl fluoride II, with the formation of a lactosyl-enzyme intermediate and liberation of hydrogen fluoride. Then the lactosyl unit is transferred to the acceptor, indolethyl cellobioside V, yielding tetrasaccharide VI. In this scheme, the major competing reaction is the simple hydrolysis of the sugar donor and of the sugar acceptor. High substrate concentrations usually favor transglycosylation reactions. In some cases, the addition of an organic solvent to the reaction medium can help transglycosylation. In fact, early experiments involving the incubation of H. insolens endoglucanase I with β-lactosyl fluoride II and indolethyl β-cellobioside V in buffer only resulted in the hydrolysis of both donor and acceptor (data not shown). Various organic solvents have thus been assayed to find good conditions for the transglycosylation reaction. With the present system, we found that a 1.5:1 (v/v) mixture of acetonitrile and maleate buffer (0.05 M, pH 7.0) gave good results. Although the presence of an organic solvent appears necessary for the transglycosylation, these conditions are detrimental for the activity of the enzyme, which showed progressive denaturation after 15–20 min. The problem was circumvented by the addition of fresh enzyme during the reaction. Finally, various donor/acceptor concentrations have been explored, and the best results were obtained with donor and acceptor concentrations of 60 and 20 mM, respectively. Using the above conditions, the monofunctionalized tetrasaccharide VI was isolated with a yield of 60%. Small proportions of other oligosaccharides deriving from side reactions were also readily isolated (Table I). The bifunctionalized tetrasaccharide IX was thus obtained with a yield of 60% from tetrasaccharide VII and an overall yield of 14% from cellobiose octaacetate. The 1H NMR spectrum of tetrasaccharide IX is shown in Fig. 2.

**Kinetic Assays**—The efficiency of intramolecular resonance energy transfer depends on the distance between donor and acceptor and on the spectral overlap between the donor emission and the acceptor absorption (23). Among the different donor-acceptor pairs potentially useful for energy transfer measurements, the pair chosen in our study was indolethanol-EDANS, for which the efficiency of the resonance energy transfer appeared satisfactory. Indolethanol absorbs at \( \lambda_{\text{max}} = 290 \) nm and emits at \( \lambda_{\text{max}} = 365 \) nm; EDANS absorbs at \( \lambda_{\text{max}} = 340 \) nm and emits at \( \lambda_{\text{max}} = 490 \) nm (Fig. 3). In addition, these two chromophoric groups can be linked to oligosaccharides via stable bonds (9).

The first important experiment was to verify the intramolecular resonance energy transfer. When the bifunctionalized tetrasaccharide IX (5–20 \( \mu \)M) was excited at 290 nm, the EDANS fluorescence recorded at 490 nm was five times higher than the resulting tetrasaccharide represents a convenient site for the introduction of the second fluorescent group via the regioselective oxidation of the C-6 position by galactose oxidase and subsequent reductive amination.

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that of a solution of free EDANS at the same concentration. On the other hand, the resonance energy transfer was found to decrease the fluorescence of the indolethyl moiety at 365 nm by 50% compared with the fluorescence of the monofunctionalized tetrasaccharide VII (Fig. 4). These values, which demonstrated the resonance energy transfer, also indicated that enzymatic hydrolyses would be monitored more sensitively by the decrease of the fluorescence at 490 nm than by the increase of the fluorescence at 365 nm.

The substrate concentrations used must be sufficiently low to prevent intermolecular resonance energy transfer. The fluorescence intensity at 490 nm of a 20 μM solution of free EDANS was found to be almost identical to that of a solution containing EDANS and indolethyl tetrasaccharide VII at the same concentration, demonstrating that intermolecular transfer can be neglected at this concentration. The kinetic assays have therefore been performed with substrate concentrations ranging from 2 to 20 μM. Recording the fluorescence intensity of the monofunctionalized tetrasaccharide IX at different concentrations (5, 10, 15, and 20 μM) for 1 min demonstrated that the fluorescence intensity at 490 nm is proportional to the substrate concentration. The decrease in fluorescence observed during enzymatic hydrolysis (an example is given in Fig. 5) is therefore directly related to the rate of hydrolysis since for each mol of substrate which is cleaved, the total fluorescence is decreased by the intensity caused by 1 mol of EDANS. Initial rates and therefore kinetic parameters are directly available from the slope of the straight lines recorded.

Four fungal recombinant cellulases from the fungus H. insolens have been tested for their ability to hydrolyze the bifunctionalized tetrasaccharide IX. These enzymes were two endoglucanases, EGI and EGV, and two cellobiohydrolases, CBHI and CBHII (10). The values of $K_m$ and $k_{cat}$ obtained for the various enzymes are presented in Table II.

EGV is the only enzyme with which no hydrolysis was detected. This result is compatible with earlier observations that EGV contains at least six subsites and that the catalytic constants for the hydrolysis of cellopentaol are very poor with a $K_m$ above 1,000 μM and a $k_{cat} = 0.84$ s$^{-1}$ (10). On the other hand, hydrolysis of cellohexaol by EGV was much more efficient ($K_m = 52$ μM and $k_{cat} = 14$ s$^{-1}$) (10). The bifunctionalized tetrasaccharide IX is probably too short, and/or the chromophoric groups perhaps prevent the productive binding of the substrate to the enzyme.

The second enzyme tested was EGI, the enzyme used for the enzymatic synthesis of the substrate. EGI proved to be very efficient for the hydrolysis of the bifluorescent substrate IX with values of $k_{cat}$ (27 s$^{-1}$) and $k_{cat}/K_m$ (3.4 s$^{-1}$ μM$^{-1}$) comparable to the values of 40 s$^{-1}$ and 2.9 s$^{-1}$ μM$^{-1}$, respectively, obtained with the same enzyme using cellopentaol as substrate (10). This observation shows that the large fluorogenic groups introduced on the tetrasaccharide have little influence on the hydrolysis of the substrate by this enzyme.

Surprisingly, the two cellobiohydrolases, CBHI and CBHII core, were also able to hydrolyze the bifunctionalized substrate with $K_m$ values in the micromolar range (Table II). The kinetic parameters show, however, that these two enzymes are less efficient than EGI since their catalytic constants were found to be, respectively, 0.40 and 0.023 s$^{-1}$ (Table II). The value of 0.40 s$^{-1}$ obtained with CBHI, albeit low, is comparable to the value of 0.96 s$^{-1}$ obtained during the hydrolysis of cellopentaol (10). This result indicates that the two fluorogenic groups of the
substrate do not affect very significantly hydrolysis of the substrate by CBHI. CBHII core is also able to cleave the bifunctionalized tetrasaccharide, although with a catalytic constant largely reduced compared with that observed against cellobeta-tioT (0.67 s⁻¹) (10). The sites of cleavage of the bifunctionalized substrate by EGI, CBHI, and CBHII were analyzed by thin layer chromatography (data not shown). EGI and CBHI were found to be able to hydrolyze the substrate at two points, whereas CBHII could cleave only one of the interosidic bonds (Fig. 6).

CBHI and CBHII from *H. insolens* display high sequence similarity with CBHI and CBHII from *Trichoderma reesei* (63 and 58% sequence identity, respectively), whose three-dimensional structures have been determined (24, 25). The only feature common to these otherwise dissimilar protein structures is the topology of their active sites. In both cases, long loops cover part of the active site cleft, and the resulting tunnel enables the threading of a cellulose chain (25). The tunnel-shaped active site of cellulobiodyrolases permits the liberation of the cellobiose product while maintaining the cellulose chain bound to the enzyme. This active site topology allows the processive, recurrent hydrolysis of cellulose chains into cellobiose (26). An important question remains with the site of the initial attack of cellulase. Three possibilities exist: (i) entry of the substrate chain end from only one entrance of the tunnel resulting in a selectivity for a single chain end type; (ii) entry of reducing chain ends from one side of the tunnel while non-reducing chain ends could enter from the other side; and (iii) a sporadic opening of the loops to allow binding of a cellulose chain in the active site. Current models favor the first possibility with a selective chain end attack for cellulobiodyrolases, with CBHI starting from the reducing ends (27, 28) and CBHII starting from the non-reducing ends of cellulose chains (28, 29). The fact that the two recombinant cellulobiodyrolases we have assayed are able to degrade the bifunctionalized substrate carrying non-carbohydrate substituents at both ends argues against the specific chain end recognition by cellulobiodyrolases and suggests that a different model for the initial attack should be envisioned.

In conclusion, the use of the transglycosylating activity of the recombinant endoglucanase EGI from *H. insolens* has provided a rapid and efficient synthesis of a bifunctionalized tetrasaccharide from two modified disaccharides. This chemoenzymatic approach has led to the preparation of a useful substrate for the biochemical characterization of cellulases since it provides a very sensitive assay that can be monitored continuously. Furthermore, the comparison of catalytic constants with those obtained on reduced cellobextrins shows that the fluorophores do not seem to influence the enzymatic hydrolysis by cellulases having a low number (<5) of subsites. For cellulases such as *H. insolens* EGV which have a large number of subsites (>5), the synthesis of a longer oligosaccharide should be envisaged. Since the efficiency of the resonance energy transfer depends both on the distance between the two chromophores and on the spectral overlap of donor and acceptor, the use of another donor-acceptor pair that would show a better spectral overlap should be considered, to compensate for the increased distance between the two fluorophores when one or two extra glucosyl residues are added. In addition to providing a sensitive assay for endoglucanases, the bifunctionalized tetrasaccharide was also found to be hydrolyzed by two cellulobiodyrolases, suggesting that these enzymes do not specifically require a free chain end for their action. Work is currently under way in our laboratory to test this hypothesis further.

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