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p-Aminophenyl 1-thio-β-D-cellobioside: Synthesis and application in affinity chromatography of exo-type cellulases

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Abstract

p-Aminophenyl 1-thio-β-D-cellobioside (APTC) is shown to be a functional affinity ligand for the separation of exo-(cellobiohydrolases) and endo-(endoglucanases) acting cellulases. APTC is prepared by direct attachment of *p*-aminobenzenethiol to 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl bromide, and subsequent deacetylation. APTC has been coupled to *N*-hydroxysuccinimide- activated agarose for affinity chromatography. *Trichoderma reesei* cellulases were used as representative enzymes. The behavior of these enzymes on APTC-affinity columns was essentially equivalent to that reported for the same enzymes on *p*-aminobenzyl 1-thio-β-D-cellobioside (ABTC)-columns; ABTC being the traditional ligand for affinity chromatography of exocellulase. The major cellobiohydrolases are retained on these columns, whereas the major endoglucanases are not. The cellobiohydrolases may be eluted from the columns by the addition of cellobiose to the mobile phase. The primary advantage of the APTC-ligand over other affinity ligands is its ease of preparation; the preparation of APTC requires approximately one-half the number of synthetic steps as required for the preparation of ABTC. © 1997 Elsevier Science Ltd.

Keywords: *p*-Aminophenyl 1-thio-β-D-cellobioside; Cellobiohydrolase; Affinity; Trichoderma

1. Introduction

Hydrolases common to microbial cellulase systems include endoglucanases (E.C. 3.2.1.4), cellobiohydrolases (E.C. 3.2.1.91) and β-glucosidases (E.C. 3.2.1.21). These enzyme classes can be effectively separated by affinity chromatography [1–3]. *p*-Aminobenzyl 1-thio-β-D-cellobioside (ABTC) is currently the most commonly used complementary ligand for this application [2]. In the ABTC-system, the major exo-acting enzymes are retained on the ABTC-support, while the major endo-acting enzymes are not.

In this note we demonstrate the usefulness of *p*-aminophenyl 1-thio-β-D-cellobioside (APTC) as an alternative affinity ligand for the separation of cellulolytic enzymes. APTC is easier to prepare than

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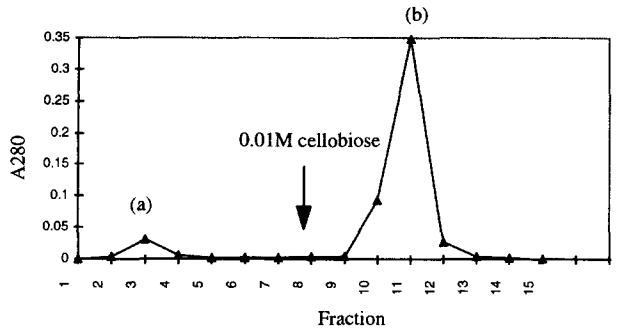

Fig. 1. APTC-derivatized affinity chromatography of CBH I. CBH I fraction obtained from DEAE–Sepharose column was applied to an APTC-derivatized affinity column, and eluted with 0.01 *M* cellobiose in 0.1 *M* sodium acetate buffer, pH 5, containing 1 mM D-glucono-δ-lactone. Peak a: unretarded CBH I (endoglucanases); Peak b: CBH I.

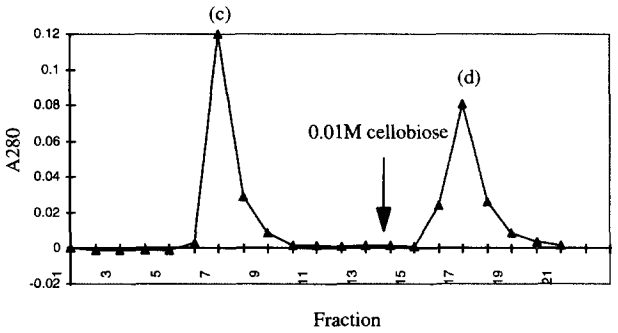

Fig. 2. APTC-derivatized affinity chromatography of CBH II. CBH II fraction obtained from DEAE–Sepharose column was applied with 0.2 *M* glucose to an APTC-derivatized affinity column, and eluted with 0.01 *M* cellobiose in 0.1 *M* sodium acetate buffer, pH 5, containing 1 m*M* D-glucono-δ-lactone. Peak c: unretarded CBH II (β-glucosidase + endoglucanases); Peak d: CBH II.

ABTC, and its interaction with exo- and endo-acting cellulases is shown to be essentially the same as that reported for ABTC.

2. Results and discussion

The synthetic approach detailed in the Experimental section provides a relatively simple means of obtaining an affinity ligand for exo-acting cellulases. The glycosyl bromide (**2**) of commercially available cellobiose octaacetate (**1**) is prepared in step one. This intermediate is then reacted with commercially available *p*-aminobenzenethiol, yielding peracetylated APTC (**3**). APTC (**4**) is then prepared by deacetylation in basic methanol. The total synthesis requires only three steps, which is a significant reduction compared to the six steps required for the synthesis of ABTC. The overall synthetic yield of **4** was 45%, which compares favorably to the 22% yield reported for the synthesis of ABTC [4]. Amino-containing ligands, such as APTC, can be readily coupled to a variety of commercially available supports [5]. In this work the APTC ligand was coupled to NHS-activated agarose. The resulting APTC-derivatized agarose supports had maximum binding capacities, based on tests with *Trichoderma reesei* cellobiohydrolase I (CBH I), of approximately 4–5 mg protein per mL gel.

Table 1
Enzymatic activities of affinity-purified cellobiohydrolases

Enzyme	β-Glucosidase (mg *p*-nitrophenol min^{-1} mg $protein^{-1}$)	HECase [a] (mg reducing sugar min^{-1} mg $protein^{-1}$)	Avicelase [b] (mg reducing sugar h^{-1} mg $protein^{-1}$)
Crude cellulase	0.029	0.270	1.674
CBH I preparation from anion-exchange column	0.001	0.059	0.294
CBH II preparation from anion-exchange column	0.065	0.151	0.368
CBH I / affinity column [c]			
Unretarded CBH I (peak a)	0.003	0.227	0.004
CBH I (peak b)	0.000	0.016	0.126
CBH II / affinity column [d]			
Unretarded CBH II (peak c)	0.109	0.380	0.065
CBH II (peak d)	0.000	0.024	0.189

[a] Hydroxyethylcellulose was used to determine endocellulase activity.
[b] Avicel was used to determine exocellulase activity.
[c] See Fig. 1.
[d] See Fig. 2.

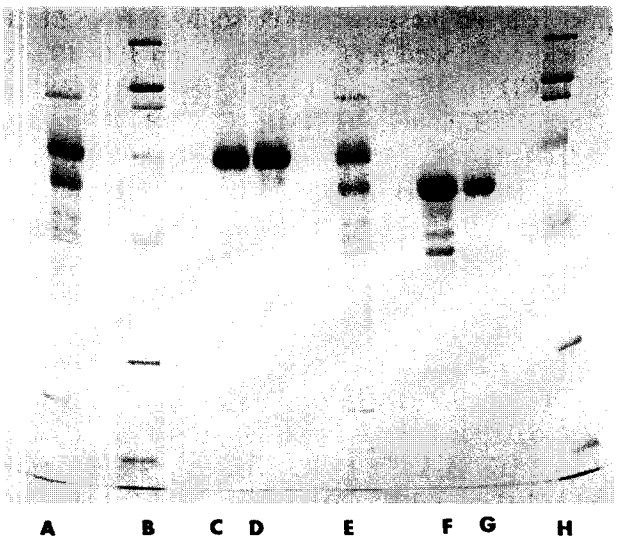

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cellulase before and after purification. Lanes A and E: crude cellulase; Lanes B and H: Protein standard; Lane C: CBH I received from APTC-derivatized affinity and HIC columns; Lane D: CBH I received from APTC-derivatized affinity column; Lane F: CBH II received from APTC-derivatized affinity column; Lane G: CBH II received from APTC-derivatized affinity and HIC columns.

In a typical CBH purification protocol [2], a crude source of CBH is initially fractionated by DEAE–anion-exchange chromatography. Fractions containing exo-cellulase activity are then affinity chromatographed to further separate exo-cellulases from contaminating non-cellulolytic proteins, endo-cellulases and β-glucosidases. This protocol was first applied to the purification of exo-acting cellulases from *Trichoderma reesei* [1]. The *Trichoderma reesei* enzyme system contains two principal exo-acting cellulases, CBH I and cellobiohydrolase II (CBH II) [6]. CBH I and CBH II are readily separated in the initial DEAE–anion-exchange purification step [7]. However, the CBH I and CBH II preparations resulting from DEAE chromatography are generally not functionally pure, due to coelution of nearly homologous endo-acting enzymes [8]. Affinity chromatography is used for the separation of these nearly homologous CBH/endo-enzyme pairs.

Figs. 1 and 2 demonstrate how the APTC-ligand may be used for affinity-based separation of exo- and endo-acting cellulases. CBH I and CBH II preparations obtained from DEAE chromatography were applied to an APTC-derivatized column. Non-retained proteins were washed through with buffer; and CBHs were then eluted by addition of cellobiose to the mobile phase. Experiments with purified endoglucanases and β-glucosidases showed that these enzymes were not retained on the affinity column. The activities of the affinity-purified CBH preparations demonstrate their improved functional purity (Table 1). The electrophoretic purity of the two cellobiohydrolases resulting from APTC-derivatized affinity chromatography is shown in lanes D and F of Fig. 3. The relatively weak staining bands of lower molecular weight are due to the presence of catalytically active exocellulase core fragments that result from proteolysis of intact enzymes [6]. The core fragments can be separated from the intact enzymes by hydrophobic interaction chromatography (HIC); the intact enzymes are retained on the column while the core fragments pass through with the void volume [9]. The electrophoretic purity of the two intact cellobiohydrolases, following HIC, is shown in lanes C and G of Fig. 3. The activities of the purified CBHs are summarized in Table 2.

In conclusion, APTC-derivatized affinity columns appear to have the same chromatographic properties as those reported for the traditional ABTC-derivatized columns and, thus, could be used interchangeably. The primary advantage of using APTC-derivatized columns lies in the relative ease of ligand synthesis.

Table 2
Enzyme activity of purified cellobiohydrolases after anion-exchange, affinity and hydrophobic interaction chromatography

Enzyme	β-Glucosidase (mg *p*-nitrophenol min^{-1} mg $protein^{-1}$)	Endocellulase [a] (mg reducing sugar min^{-1} mg $protein^{-1}$)	Exocellulase [b] (mg reducing sugar h^{-1} mg $protein^{-1}$)
Crude cellulase	0.027	0.300	1.580
CBH I	0.000	0.001	0.205
CBH II	0.000	0.017	0.209

[a] Hydroxyethylcellulose was used to determined endocellulase activity.
[b] Avicel was used to determined exocellulase activity.

3. Experimental

General methods.—IR spectra of intermediates were obtained using a Nicolet-510P spectrophotometer. The ^{1}H NMR spectra were recorded at 400 MHz with a Bruker AM 400 spectrometer, using tetramethylsilane as an internal standard. Assignments were confirmed by double resonance irradiation and 2D-COSY experiments. Enzyme-containing fractions resulting from chromatography were identified by their UV absorbance (Shimadzu UV 160U spectrophotometer).

*Synthesis of p-aminophenyl 1-thio-β-*D*-cellobioside (APTC)* (**4**).—*2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-β-*D*-glucopyranosyl bromide* (**2**). HBr (30 mL of a 30% wt solution in acetic acid) was added to a cold solution containing 2.00 g (2.95 mmol) α-D-cellobiose octaacetate (**1**) dissolved in 25 mL of dichloromethane, and the resulting solution was stirred for 5 h at 0 °C. The mixture was then diluted with 50 mL of dichloromethane and successively washed with equal volumes of ice-cold aqueous 10% $KHSO_4$ (2 ×), aqueous saturated $NaHCO_3$ (2 ×), and water (2 ×). The washed dichloromethane solution was dried over anhydrous Na_2SO_4 and evaporated at room temperature under reduced pressure to give compound **2** (2.05 g, 99%): mp 182–184 °C, lit. 185 °C [10].

p-*Aminophenyl 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl) -β-*D*-glucopyranoside* (**3**).—Glycosyl bromide (**2**) (approximately 2 g, 2.9 mmol) was dissolved in 80 mL of acetone; and the resulting solution was mixed with 56 mL of an aqueous solution containing 1.76 g (12.7 mmol) K_2CO_3 and 1.6 g (12.7 mmol) *p*-aminobenzenethiol. This reaction mixture was stirred overnight at room temperature. The solvent was subsequently removed by evaporation at room temperature under reduced pressure. The resulting residue was dissolved in 100 mL of dichloromethane; the dichloromethane solution was then washed, dried and evaporated as described above. The target compound was purified by silica gel chromatography (70–230 mesh, SIGMA Chemical Co., St. Louis, MO) using a 2.5 × 40 cm column, and an EtOAc:hexane (1:1 v/v) eluent. The compound of interest was identified by thin-layer chromatography using silica plates (LK6DF, Whatman Inc., Clifton, NJ) and the same mobile phase used for column chromatography; spots were detected by *p*-anisaldehyde- (sugar) and ninhydrin-based (amine) visualizing reagents [11]. Fractions containing the target compound were pooled, evaporated to dryness under reduced pressure, and crystallized from MeOH to yield compound **3** (1.1 g, 53%): mp 190–193 °C; IR (NaCl): ν 1757 and 1226 (ester) and 3468 and 3395 cm^{-1} (amine); ^{1}H NMR ($CDCl_3$): δ 1.97–2.1 (s, 21 H, OAc), 3.55 (m, 1 H, H-5), 3.64 (m, 1 H, H-5′), 3.69 (m, 1 H, H-4), 3.78 (s, 2 H, NH), 4.0 (dd, 1 H, $J_{5'6a'}$ 1.9 Hz, $J_{6a'6b'}$ 12.5 Hz, H-6a′), 4.05 (dd, 1 H, J_{56a} 5.1 Hz, J_{6a6b} 11.9 Hz, H-6a), 4.35 (dd, 1 H, $J_{5'6b'}$ 4.1 Hz, $J_{6a'6b'}$ 12.5 Hz, H-6b′), 4.48 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 4.5 (d, 1 H, $J_{1'2'}$ 10.2 Hz, H-1′), 4.58 (dd, 1 H, J_{56b} 1.4 Hz, J_{6a6b} 11.9 Hz, H-6b), 4.8 (t, 1 H, $J_{2,3}$ 9.7 Hz, H-2), 4.9 (t, 1 H, $J_{2'3'}$ 8.5 Hz, H-2′), 5.05 (t, 1 H, $J_{4'5'}$ 9.6 Hz, H-4′), 5.1 (t, 1 H, $J_{3'4'}$ 9.2 Hz, H-3′), 5.15 (t, 1 H, $J_{3,4}$ 9.2 Hz, H-3), 6.5 (d, 2 H, *J* 8.2 Hz, ArH), and 7.27 (d, 2 H, *J* 8.2 Hz, ArH).

p-*Aminophenyl 1-thio-β-*D*-cellobioside* (**4**).—The protected thioglycoside (**3**) was deacetylated by treatment with NaOMe in MeOH as described by Thompson and Wolfrom [12] to yield compound **4** (0.26 g, 85%): mp 247–250 °C. Complete deacetylation was confirmed by the absence of acetyl proton resonances in NMR experiments. ^{1}H NMR (Me_2SO-d6): δ 3.0 (m, 6 H,), 3.24 (d, 1 H, *J* 5.8), 3.3 (m, OH), 3.65 (m, 4 H), 4.25 (d, 1 H, *J* 9.6), 4.25 (d, 1 H, *J* 6), 4.5 (m, 2 H), 4.7 (d, 1 H), 5 (dd, 2 H, *J* 11.6), 5.2 (dd, 4 H), 6.50 (d, 2 H, *J* 8.4 Hz, ArH), and 7.20 (d, 2 H, *J* 8.4 Hz, ArH).

Separation of Trichodermareesei *cellobiohydrolases.—Anion-exchange chromatography. Trichoderma reesei* crude cellulase (Spezyme™-CP, Environmental BioTechnologies Inc., Menlo Park, CA) was initially fractionated by DEAE–Sepharose chromatography (Pharmacia Inc., Piscataway, NJ) as described by Beldman et al. [13]. The resulting CBH I and CBH II preparations were used for affinity chromatography experiments.

Affinity chromatography.—APTC was coupled to Affigel 10 (BioRad Laboratories, Hercules, CA) as suggested by the manufacturer. Derivatized gels were packed in silica glass columns (1 × 10 cm). CBH I and CBH II preparations resulting from DEAE chromatography were further chromatographed on the APTC-derivatized affinity columns. The initial mobile phase for CBH I chromatography was 0.1 *M* NaOAc, pH 5, containing 1 m*M* D-glucono-δ-lactone; the initial mobile phase for CBH II chromatography was the same as that for CBH I with the exception that the solution also contained 0.2 *M* glucose. All solutions were 1 m*M* in D-glucono-δ-lactone in order to retard ligand hydrolysis in the presence of β-glucosidases [2]. Mobile phase flow

rates were approximately 0.5 mL/min and fractions were collected every 10 min. Adsorbed CBHs were eluted from the affinity columns by making the starting buffer 0.01 *M* in cellobiose.

Hydrophobic interaction chromatography.—CBH I- and CBH II-containing fractions from APTC-derivatized affinity chromatography were chromatographed on a phenyl Sepharose FF column (Pharmacia Inc., Piscataway, NJ) (1 × 10 cm) to separate intact enzymes from catalytic-core fragments. CBH I and CBH II were applied to the columns in 25 m*M* NaOAc buffer at pH 5, containing either 0.85 or 0.35 *M* ammonium sulfate, respectively. Columns were washed with starting buffer prior to eluting enzymes with a gradient of 0.85–0.35 *M* ammonium sulfate (for CBH I) or 0.35–0.01 *M* ammonium sulfate (for CBH II) in 25 m*M* NaOAc buffer, pH 5.

Electrophoresis.—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using 10% acrylamide gels [14].

Activity measurements.—Endo-type cellulase activity was measured with either carboxymethyl cellulose (CMC; CMC7HOF, Aqualon Co., Wilmington, DL) or hydroxyethyl cellulose (HEC; medium viscosity, Fluka Chemical Corp., Ronkonkoma, NY) substrates. Enzyme solution (0.2 mL) was added to 0.8 mL of 0.5% CMC, or 1.8 mL of 0.5% HEC, in 50 m*M* NaOAc buffer, pH 5. Enzyme-substrate mixtures were incubated at 50 °C for 30 min. Reactions were terminated by boiling; new reducing ends were estimated by the alkaline–copper assay of Nelson [15]. Results are reported in units of mg reducing sugar min^{-1} mg $protein^{-1}$.

Exo-type cellulase activity was measured with a microcrystalline cellulose (MCC; Avicel PH 101, FMC Cor. Philadelphia, PA) substrate. Enzyme solution (0.3 mL) was added to 1.5 mL 50 m*M* NaOAc buffer, pH 5, containing 1.8 mg MCC. Enzyme–substrate mixtures were incubated at 50 °C for 4 h with orbital shaking at 160 rpm. Reactions were terminated by centrifuging to pellet the insoluble MCC, and then removing the soluble phase. Total reducing sugar liberated into the soluble phase was then determined [15]. Results are reported in units of mg reducing sugar h^{-1} mg $protein^{-1}$.

β-Glucosidase activities were determined at 50 °C by measuring rates of *p*-nitrophenyl-β-D-glucoside hydrolysis as described by Wood and Bhat [16].

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