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p-Aminophenyl β-cellobioside as an affinity ligand for exo-type cellulases

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Abstract

p-Aminophenyl β-cellobioside (PAPC) is shown to be an effective affinity ligand for the chromatographic fractionation of cellobiohydrolases (CBHs). A crude cellulase preparation from the filamentous fungus *Trichoderma reesei* served as a representative source of enzymes for this study. Prior to chromatography, PAPC was tethered via its amino functional group to *N*-hydroxysuccinimide-activated agarose. The resulting affinity matrix specifically retained the CBH component of relatively complex cellulase mixtures. The purity of the resulting CBH preparations, based on measured specific activities, was comparable to that of corresponding enzyme preparations obtained using more traditional thioglycoside-based affinity ligands. The application of PAPC as an affinity ligand illustrates that the tethered ligand associates with the *T. reesei* CBHs in a catalytically nonproductive mode. In contrast, the free ligand is readily hydrolyzed by *T. reesei* CBH I.

Keywords: *p*-Aminophenyl β-cellobioside; Cellobiohydrolase; Affinity; Ligand

1. Introduction

Microbial cellulolytic enzyme systems capable of degrading crystalline cellulose typically contain at least three classes of enzymes: endoglucanases (EC 3.2.1.4), cellobiohydrolases (CBHs) (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Studies aimed at elucidating the properties of the individual enzymes comprising these mixtures are often hampered by the difficulties encountered when trying to obtain functionally pure enzymes. Affinity chromatography has proven to be a most valuable approach to the separation of nearly homologous endo- and exo-acting cellulases [1,2]. *p*-Aminobenzyl 1-thio-β-D-cellobioside (ABTC) was the first cello oligo saccharide-based affinity ligand used for the fractionation of endo- and exo-cellulases [3]. Other cello oligo saccharide derivatives have subsequently been used for the same application. Orgeret et al. [4] introduced *p*-aminophenyl 1,4-dithio-β-D-cellobioside as an alternative to ABTC, the advantage of this ligand being that the $1\rightarrow4$ thioglycosidic linkage is resistant to β-glucosidase-catalyzed hydrolysis. Piyachomkwan et al. [5] then demonstrated the use of *p*-aminophenyl 1-thio-β-cellobioside (APTC) for the fractionation of endo- and exo-acting cellulases, the advantage of this ligand being the relative ease with which it can be prepared. The common feature of all of these ligands is the substitution of an S-linkage for an O-linkage at the aglyconic bond of the cellobioside derivative, thus making all of these ligands resistant to exo-cellulase (CBH)-catalyzed hydrolysis.

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Chromogenic derivatives of cellobioside, e.g., nitrophenyl glycosides, 4-methylumbelliferyl glycoside, etc., have long been used as model substrates for the study of CBHs [6–8]. These substrates are susceptible to CBH-catalyzed hydrolysis at the agluconic linkage, although CBHs differ markedly in their ability to catalyze this reaction [9]. Considering the *Trichoderma reesei* cellulase system, CBH I catalyzes this reaction and CBH II does not. Thus far, these compounds have only been considered as potential substrates for the CBHs. However, the following two observations suggest that compounds of this type have potential as affinity ligands for cellulase fractionation. First, CBH II does not catalyze the hydrolysis of these compounds, and yet the enzyme clearly associates with their immobilized thio analogs, e.g., ABTC [3] and APTC [5]. Second, although these compounds are substrates for CBH I, this enzyme preferentially acts at the reducing terminus of cellooligosaccharide chains [10–12]; so the reducing-end-tethered form of these substrates (as used in affinity chromatography) would not necessarily form a catalytically productive complex.

The intent of this communication is to demonstrate that amino terminus-tethered *p*-aminophenyl β-cellobioside (PAPC) associates with the major *T. reesei* CBHs in a catalytically nonproductive manner, thus making it a convenient affinity ligand. This finding has implications with respect to the mode of binding of similar thio analogs that are currently used as affinity ligands for exo-acting cellulases.

2. Results and discussion

As expected [8,9], CBH I catalyzed the hydrolysis of the aglyconic linkage in *p*-nitrophenyl β-cellobioside and CBH II did not. Neither enzyme catalyzed the hydrolysis of the compound's interglycosidic linkage. The two enzymes showed the same specificities with PAPC, the reduced form of *p*-nitrophenyl β-cellobioside. However, coupling of PAPC, via its amino functional group, to an agarose matrix rendered it resistant to hydrolysis by

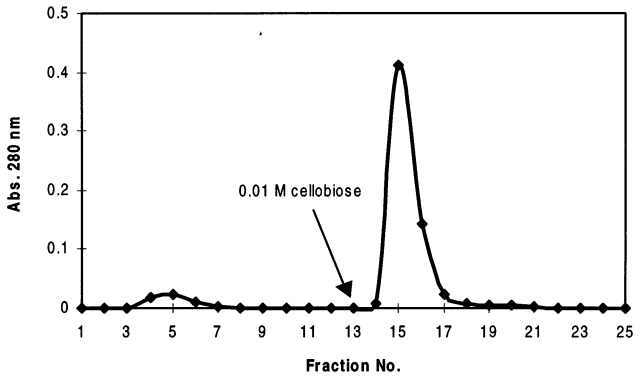

Fig. 1. Affinity chromatography of CBH I using PAPC-derivatized agarose. A partially purified CBH I preparation was applied to the PAPC-affinity column in 0.1 M NaOAc, pH 5 containing 1 mM gluconolactone. CBH I was eluted by making the mobile phase 0.01 M cellobiose.

either of the CBHs. CBH I and CBH II exhibited no measurable activity toward either of the *O*-glycosidic linkages in tethered PAPC over the course of a 37-h reaction period at 50 °C, pH 5 (optimum conditions for CBH activity).

To test the application of PAPC as an affinity ligand for the fractionation of CBHs, partially purified CBH I and CBH II were chromatographed using PAPC-coupled agarose as the stationary phase (see chromatograms in Figs. 1 and 2). In both cases, the CBHs were retained on the stationary phase while non-CBH proteins passed through the column. The CBHs were eluted from the column only after making the mobile phase 0.01 M cellobiose. The electrophoretic purity of the adsorbed CBHs was essentially the

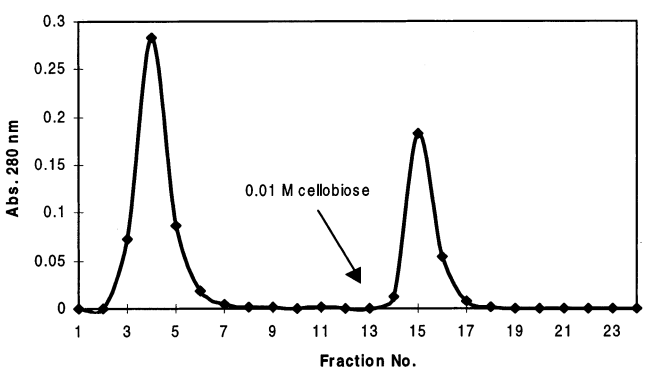

Fig. 2. Affinity chromatography of CBH II using PAPC-derivatized agarose. A partially purified CBH II preparation was applied to the PAPC-affinity column in 0.1 M NaOAc, pH 5 containing 1 mM gluconolactone and 0.2 M glucose. CBH II was eluted by making the mobile phase 0.01 M cellobiose.

Table 1
Specific activities of purified CBH fractions

Enzyme	Specific activities		
	β-Glucosidase[a] (μmol *p*-nitrophenol min^{-1} mg $protein^{-1}$)	Endo-cellulase[b] (μmol reducing sugar min^{-1} mg $protein^{-1}$)	Exo-cellulase[c] (μmol reducing sugar min^{-1} mg $protein^{-1}$)
Crude cellulase	0.125	0.279	0.036
Anion-exchange column			
CBH I fraction	0.001	0.099	0.013
CBH II fraction	0.373	0.470	0.019
CBH I/affinity column			
APTC[d]	0	0.014	0.011
PAPC[e]	0	0.011	0.010
CBH II/affinity column			
APTC[d]	0	0.034	0.011
PAPC[e]	0	0.030	0.011

[a] *p*-Nitrophenyl β-D-glucopyranoside was used to determine β-glucosidase activity.
[b] Hydroxyethylcellulose was used to determine endocellulase activity.
[c] Avicel was used to determine exocellulase activity.
[d] Activity of purified CBH I (or CBH II) after anion-exchange and APTC-affinity column; APTC was synthesized as described by Piyachomkwan et al. [5].
[e] Activity of purified CBH I (or CBH II) after anion-exchange and PAPC-affinity column.

same as that of the purified enzymes prepared using APTC-derivatized agarose [5]. The relative functional purity of the resulting CBH preparations was evaluated by comparing their specific activities with those of analogous preparations resulting from the more traditional aryl thioglycoside-based affinity chromatography [5]. The specific activities of the CBH I and CBH II preparations resulting from PAPC-based affinity chromatography were found to be similar to those obtained from aryl thioglycoside-based affinity chromatography (Table 1). The preparations had no detectable β-glucosidase activity, and their corresponding endo-type activities decreased dramatically. The decrease in the specific activity of the two CBH preparations toward microcrystalline cellulose is caused by the relative absence of synergism that results from enzyme fractionation [13].

The mechanistic explanation for the lack of activity of CBH I on amino terminus-tethered PAPC is not obvious. CBH I is a retaining exo-cellulase which preferentially acts at the reducing end of cellooligosaccharide chains [10–12]. The obvious difference between tethered and free PAPC is that the terminus analogous to the reducing end, the amino terminus in this case, is sterically blocked only in the case of the tethered ligand. The implication of this is that the tethered ligand must back into the active site tunnel via the opening which is thought to primarily serve as an exit for generated product. Free PAPC may enter the active site tunnel via either end. CBH I is known to have ten well-defined glucosyl binding subsites within its active site tunnel [14], and cellobiose is known to preferentially bind at the product binding subsites (subsites $+1$ and $+2$, as described by Stahlberg et al. [15]). It is likely that the two glucosyl units of the tethered ligand also associate with the product-binding subsites of CBH I. In contrast, the glucosyl units of free PAPC, when positioned for hydrolysis, are presumably bound in subsites -1 and -2, with the aminophenyl group being aligned at subsite $+1$. Tethered PAPC may not be able to form the analogous catalytically productive complex, covering subsites -2 to $+1$, due to

steric limitatations arising from the bulky matrix to which it is affixed. A second possibility is that a kinetic barrier restricts the activity of CBH I on tethered PAPC. This scenario may result as a consequence of the ligand backing into the active site tunnel, as required for the tethered form. In this case, the CBH I–ligand complex would have been formed without the ligand ever passing through the catalytically productive complex (-2 to $+1$).

In this study PAPC was prepared in four synthetic steps. However, PAPC can be obtained relatively easily by the reduction of commercially available *p*-nitrophenyl β-cellobioside (PNPC). PNPC is relatively inexpensive, currently selling for ca. US$60 per 100 mg (Sigma Chemical Co., St. Louis, MO), and PAPC yields following PNPC reduction are typically $>95\%$. The affinity supports demonstrated in this work were prepared in coupling reaction mixtures containing ca. 10 mg of ligand per mL of agarose gel. This resulted in affinity gels having maximum CBH I ($\varepsilon = 73{,}000\ M^{-1}\ cm^{-1}$, $M_w = 65{,}000$ [16]) binding capacities of ca. 4 mg of protein per mL of gel. PAPC-based columns may be reused as necessary, our laboratory having now used a single column for eight chromatographic runs of *T. reesei* CBHs without observing appreciable changes in column performance. The reality that PAPC can be obtained from an inexpensive commercially available precursor in but a single, high-yielding, synthetic step makes it the most readily accessible of any of the currently available affinity ligands for this purpose.

In conclusion, this work demonstrates that the affinity ligands commonly used for the fractionation of endo- and exo-acting cellulases associate with prototypical CBHs in a catalytically nonproductive mode. Hence, in many cases there may be relatively little advantage in incorporting a thio linkage at the aglyconic bond.

3. Experimental

General methods.—All organic solutions were dried with anhydrous Na_2SO_4. Solvents were evaporated at reduced pressure (below 45 °C). The ^1H NMR spectra were recorded at 400 MHz with a Bruker AM 400 spectrometer, using tetramethylsilane as an internal standard. Reactions were monitored by TLC on precoated plates of Silica Gel 60 F_{254} (Whatman, Clifton, NJ). The following solvent systems (v/v) were used for TLC: (A) 1:1 EtOAC–hexane with 0.5% 2-propanol; (B) 5:4:1 chloroform–MeOH–H_2O. TLC separated compounds were visualized by exposure to UV light, reacting with *p*-anisaldehyde–sulfuric acid visualizing reagent, and reacting with a ninhydrin-based visualizing reagents [17].

p-*Nitrophenyl 2,3,6-tri*-O-*acetyl-4*-O-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*glucopyranosyl)-β*-D-*glucopyranoside* (**2**).—Compound **2** was prepared under phase-transfer catalyzed conditions [18]. A solution of hepta-*O*-acetylcellobiosyl bromide (**1**) [5] (1.01 g, 1.44 mmol), tetrabutylammonium hydrogen sulfate (0.49 g, 1.44 mmol) and *p*-nitrophenol (0.4 g, 2.88 mmol) in CH_2Cl_2 (10 mL) was mixed with M aq NaOH (10 mL). The mixture was stirred and gently warmed until all solids dissolved. The two-phase reaction mixture was vigorously stirred at room temperature for 3 h. TLC (solvent A) indicated the disappearance of **1** and the presence of **2**. The organic phase was successively washed with cold M NaOH (2×20 mL) and water (2×20 mL). The washed organic phase was then dried (Na_2SO_4), filtered and concentrated. A solution of residue in 1:1:1.5 EtOAc–2-propanol–CH_2Cl_2 (14 mL) was filtered through a layer of silica gel (230–400 mesh, Sigma Chemical Co., St. Louis, MO), which was washed with 1:1 EtOAc–hexane containing 0.5% 2-propanol (200 mL). The combined filtrate and washings were evaporated to dryness and crystallized from 95% EtOH to give **2** (0.48 g, 44%): mp 245–247 °C.

p-*Nitrophenyl β-cellobioside* (**3**).—The acetylated glycoside **2** (0.48 g, 0.63 mmol) was deacetylated with sodium methoxide (1 M in MeOH, 0.36 mL) in MeOH (80 mL). The mixture was stirred overnight at room temperature, neutralized with Amberlite IR-120 (H^+) resin, filtered and concentrated. Compound **3** was crystallized from MeOH (0.28 g, 95%): mp 253–255 °C, lit. 255–256 °C [19].

NMR spectra of synthesized compound **3** matched that of commercially available compound **3** (Sigma Chemical Co., St. Louis, MO).

p-*Aminophenyl β-cellobioside (PAPC)* (**4**).—Compound **4** was obtained by catalytic hydrogenation of **3** using 10% Pd on activated charcoal as a catalyst. TLC (solvent B) indicated the complete conversion of the nitro compound **3** into its amino derivative **4**.

Preparation of PAPC-derivatized agarose.—PAPC was coupled to Affigel 10 (BioRad Laboratories, Hercules, CA), under anhydrous condition to minimize non-specific protein–matrix interactions [2], as recommended by the manufacturer. The gel (10 mL), after being washed with cold 2-propanol (50 mL), was added to a solution of PAPC (200 μmol) in anhyd MeOH (15 mL). The slurry was rotated end-over-end for 3 h at room temperature. Ten milliliters of 1 M ethanolamine, pH 8, was then added to the slurry and allowed to react for 1 h in order to block active ester groups remaining on the gel. The resulting gel was washed with 0.1 M NaOAc, pH 5, containing 1 mM gluconolactone (100 mL) and loaded into 1 × 8 cm glass columns.

Protein-binding capacities of the PAPC gels were determined in partition equilibrium experiments under chromatographic conditions. Control experiments, using non-PAPC-coupled/ethanolamine-blocked gels, demonstrated negligible binding of *T. reesei* proteins to the underivatized matrix.

Affinity chromatography of cellobiohydrolases.—Crude cellulase produced by *T. reesei* (Spezyme™-CP, Environmental BioTechnologies, Menlo Park, CA) was initially fractionated by anion-exchange chromatography using DEAE-Sepharose [20]. The resulting partially purified CBH I and CBH II preparations (10–25 mg) were further fractionated using PAPC-derivatized agarose as the affinity matrix. CBH I was applied to the column in 0.1 M NaOAc, pH 5, containing 1 mM gluconolactone. CBH II was applied to the column in 0.1 M NaOAc, pH 5, containing 1 mM gluconolactone and 0.2 M glucose. Gluconolactone is included in the mobile phase for suppression of β-glucosidase activity [3]. Glucose is included in the specified mobile phases to enhance the association of CBH II with the ligand [2,21]. Chromatography was performed at 4 °C with the mobile phase flow rate at 0.5 mL min^{-1}. Adsorbed CBHs were eluted by the addition of cellobiose, 0.01 M, to the mobile phase. Protein-containing fractions were detected by monitoring absorbance at 280 nm.

Endo- and exo-type cellulase activities were measured as described previously [5] using hydroxyethylcellulose (HEC; medium viscosity, Fluka Chemical Co., Ronkonkoma, NY) and microcrystalline cellulose (MCC; Avicel PH 101, FMC, Philadelphia, PA) as substrates. The amount of reducing sugar liberated was determined by a colorimetric method (*p*-hydroxybenzoic acid hydrazide, PAHBAH) [22]. β-Glucosidase activities were measured using *p*-nitrophenyl β-D-glucopyranoside as substrate [23]. Enzyme activities were measured at 50 °C in 50 mM NaOAc buffer, pH 5.

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