

Redefining *XynA* from *Penicillium funiculosum* IMI 378536 as a GH7 cellobiohydrolase

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Abstract The secretome of *Penicillium funiculosum* contains two family GH7 enzymes, one of which (designated *XynA*) has been described as a xylanase. This is unusual because it is the only xylanase in family GH7, which is mainly composed of cellobiohydrolases and endoglucanases, and also because *XynA* is highly similar to the cellobiohydrolase I from *Talaromyces emersonii* and *Trichoderma reesei* (72 and 65 % identity, respectively). To probe this enigma, we investigated the biochemical properties of *XynA*, notably its activity on xylans and β -D-glucans. A highly pure sample of *XynA* was obtained and used to perform hydrolysis tests on polysaccharides. These revealed that *XynA* is 100-fold more active on β -1,4-glucan than on xylan. Likewise, *XynA* was active on both 4-nitrophenyl- β -D-lactopyranoside (*p*NP- β -D-Lac) and 4-nitrophenyl- β -D-cellobioside (*p*NP-cellobiose), which shows that *XynA* is principally an exo-acting type 1 cellobiohydrolase enzyme that displays 5.2-fold higher performance on *p*NP-cellobiose than on *p*NP- β -D-Lac. Finally, analyses performed using cellodextrins as substrate revealed that *XynA* mainly produced cellobiose (C2) from

substrates containing three or more glucosyl subunits, and that C2 inhibits *XynA* at high concentrations ($IC_{50}^{C2} = 17.7 \mu M$). Overall, this study revealed that *XynA* displays typical cellobiohydrolase I activity and confirms that the description of this enzyme in public databases should be definitively amended. Moreover, the data provided here complete the information provided by a previous proteomics investigation and reveal that *P. funiculosum* secretes a complete set of cellulose-degrading enzymes.

Keywords Cellobiohydrolase I · *Penicillium funiculosum* · GH7 · Cellulase · *XynA*

Introduction

Plant cell walls contain between 35 and 50 % of cellulose, a polymer composed of β -1,4 glucan chains that are organized into regular crystalline microfibrils (approximately 2.5 nm in diameter) that are embedded in a matrix of hemicellulose and lignin. To harness the glucose contained within cellulose, many microorganisms such as fungi and bacteria have devised enzymatic strategies to break down plant cell walls, thus overcoming the various obstacles that have evolved in plants to prevent this; For example, certain filamentous fungi produce potent secretomes that contain a wide variety of enzymes and accessory proteins that are able to penetrate plant cell walls and degrade cellulose microfibrils [8, 11]. Generally, cellulolytic enzyme systems contain three types of activity, which act cooperatively to bring about cellulose depolymerization: (1) endo- β -1,4-glucanase (EC 3.2.1.4) that randomly or specifically (depending on the enzyme) cleaves β -1,4-bonds between internal D-glucosyl units, (2) exo- β -1,4-glucanase or cellobiohydrolase (EC 3.2.1.91) that attacks from chain extremities to hydrolyze internal

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β -1,4-bonds and release cellodextrins, and (3) β -glucosidase (EC 3.2.1.21) that hydrolyzes cellodextrins, cleaving off glucose units from nonreducing ends [5, 9, 28]. In the CAZY classification of glycoside hydrolases (GH), such enzyme types are found in many families, but cellobiohydrolases (CBH) and many endo- β -1,4-glucanases (EG) are found in families GH5, 6, 7, 8, 9, 45, and 48 [15, 30].

Some CBHs and EGs are modular proteins, composed of a catalytic domain that is frequently, though not systematically, connected to a carbohydrate binding module (CBM) by a linker sequence that is rich in serine, threonine, and proline. These types of protein are made up of β -sheets arranged into β -sandwiches connected by loops [16]. Interestingly, CBHs and EGs possess quite similar three-dimensional (3D) structures that differ mainly in the loop regions. In CBHs, loops are generally longer and their amino acid sequence is generally different from that of EGs [10, 31]. Accordingly, the longer loops of CBHs close the substrate binding region, producing a tunnel-like configuration [2]. X-ray structure analysis of CBHs and EGs from family GH7 has revealed that the former possess approximately 11 substrate binding subsites, whereas EGs display 4–5 subsites, which form part of an open active site cleft. Overall, these differences partly explain the different mode of action of EGs and CBHs, the latter acting on β -1,4-linked glucan chains via a processive mechanism [30].

For many years, cellulases have been commercialized for a wide variety of industrial applications, which include textile modifications, detergent applications, and paper pulp refining [3]. However, one of the major focuses of research on cellulases has been the development of efficient cocktails for biorefining of lignocellulosic biomass, notably for bioethanol production [22, 33]. Similarly, the food and feed sector constitutes a considerable market for cellulase cocktails, which are used to increase the nutritional value and digestibility of cereals [1]. The main sources of industrial enzymes are microorganisms such as yeast and bacteria, although filamentous fungi are by far the most significant source, furnishing approximately 50 % of all industrial enzymes [6]. Although the filamentous fungus *Trichoderma reesei* is probably the most well-known producer of cellulolytic cocktails, other strains, such as *Penicillium funiculosum*, are also used to produce commercial cellulase cocktails that contain a wide variety of carbohydrate-acting enzymes, as revealed by an extensive proteomics analysis [13]. Among the enzymes found in the secretome of *P. funiculosum* (commercialized under the name Rovabio Excel by Adisseo S.A.S and previously by Rhodia Food UK Ltd.) are two proteins that belong to family GH7, one of which has been previously designated as *XynA* and biochemically described as a xylanase [12]. This is intriguing because *XynA* is the only known family GH7 xylanase so far described. Therefore, to follow up on

the work performed by Furniss et al. [12], we now describe a thorough biochemical analysis of *XynA*, using the native enzyme purified directly from *P. funiculosum* secretome.

Materials and methods

Chemicals and reagents

Medium-viscosity barley β -glucan (MV β G), carboxymethyl cellulose—4 M (CMC), and wheat arabinoxylan (WAX) were purchased from Megazyme (Wicklow, Ireland), while birchwood xylan (BX), 4-nitrophenyl- β -D-lactopyranoside (*p*NP- β -D-Lac), and bovine serum albumin (BSA) were purchased from Sigma Aldrich (Buchs SG, Switzerland). 4-Nitrophenyl- β -D-cellobioside (*p*NP-cellobiose) was purchased from Carbosynth (Compton, UK).

Enzyme purification

The protein *XynA* was purified to near homogeneity in three steps using a sequence of hydrophobic interaction chromatography (HIC), then ion-exchange chromatography (IEC), and finally HIC once again, starting with the culture supernatant (1,700 mL) of *P. funiculosum* IMI 378536 supplied by Adisseo SAS (Anthony, France). The initial HIC was performed on a fast protein liquid chromatography (FPLC) system (AKTA Prime) equipped with a Phenyl Sepharose 6 FF High Sub column (GE Healthcare, France) equilibrated in 50 mM sodium phosphate buffer (pH 7), containing 1.2 M $(\text{NH}_4)_2\text{SO}_4$. Proteins were eluted using a $(\text{NH}_4)_2\text{SO}_4$ gradient (1.2 to 0 M over 10 column volumes at flow rate of 100 mL/min). Subsequently, the sample was applied to a Source 15 Q anion exchange column (GE Healthcare, France), equilibrated in 20 mM Tris-HCl, pH 7, and proteins were eluted using a two-step gradient (0–500 mM NaCl over 20 column volumes at 17 mL/min, then 500 mM to 1,000 mM NaCl over 10 column volumes at 17 mL/min). Finally, to polish the purified protein sample, fractions containing the protein of interest were pooled and dialyzed against 50 mM sodium phosphate buffer pH 7, and $(\text{NH}_4)_2\text{SO}_4$ was added to final concentration of 1 M. The protein was injected onto a Phenyl FF 16/10 column (GE Healthcare, France), and then eluted using a $(\text{NH}_4)_2\text{SO}_4$ gradient (1 to 0 M over 40 min). Fractions (5 mL) were collected, dialyzed against sodium phosphate buffer 50 mM, pH 7, and then assayed for β -xylanase (BX) and β -glucanase (MV β G) activities, respectively.

Protein sequence analysis

To verify that the purified sample was *XynA*, protein sequence analysis was performed. Briefly, the purified

protein sample was first submitted to trypsin digestion, which was expected to generate fragments that would provide approximate protein sequence coverage of 50 %, and 5 µg of the sample was analyzed on a 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel running in TGS [25 mM Tris, 192 mM glycine, and 0.1 % (w/v) SDS, pH 8.3] buffer. Subsequently, nano liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed and the resulting sequences were used to interrogate the Spot Tremb database.

Measurement of enzyme activity

Enzyme activity was assayed on polysaccharides, using the dinitrosalicylic acid (DNS) method to quantify reducing sugars in the reaction medium [23]. All reactions were performed for 20 min at 30 °C in 40 mM sodium citrate buffer, pH 5, containing BSA at 1 mg/mL, using MVβG (1 % w/v), CMC (2 % w/v), and BX (4 % w/v) or WAX (1 % w/v) to assay for β-glucanase and xylanase activity, respectively. At regular intervals during reactions, samples were removed, an equal volume of DNS reagent was added (100 µL), and the mixture was incubated for 20 min at 95 °C and then for 10 min on ice. Subsequently, 1 mL ultrapure water (room temperature) was added to each sample and the optical density (OD) was read at 540 nm using a Cary 100 Bio UV-Visible spectrophotometer (Agilent technologies, Santa Clara, USA). Activity was determined using standard curves prepared using xylose or glucose as appropriate. Activities were expressed as the quantity of glucose/cellobiose or xylose equivalents (µmol) released per min per liter and per mg of *XynA*. To determine the kinetic constants (K_M and k_{cat}), *XynA* was incubated with different concentrations of MVβG (0.1–1.4 % w/v) or CMC (0.1–1.6 % w/v). Afterwards, data were analyzed using weighted nonlinear least-squares regression and a one-site saturation algorithm in SigmaPlot software version 11.0 (SPSS, USA), which generated K_M and k_{cat} values. Accounting for the fact that it was not possible to obtain an accurate estimation of the molarity of the complex polysaccharides, values for K_M were expressed as apparent values in units of mg mL⁻¹.

To measure cellobiohydrolase activity, the chromogenic substrates *p*NP-β-D-Lac and *p*NP-cellobiose were employed. Reactions were conducted at 40 °C in 40 mM sodium citrate buffer, pH 5, and the release of *p*NP was monitored in a discontinuous manner by removing samples (40 µL) at regular intervals. The enzyme reaction was stopped by adding 200 µL 1 M sodium carbonate (pH 9), and the absorbance at 402 nm was measured using a Sunrise microplate reader (TECAN, Männedorf, Switzerland). The amount of free *p*NP was determined by

comparing values against a standard curve prepared using a known range of *p*NP concentrations.

Temperature optimum and thermostability

To determine the optimum temperature for enzyme activity, reactions were performed using the conditions described above, but using different temperatures over the range 30–65 °C. Similarly, to determine thermostability, enzyme solutions in 40 mM sodium citrate buffer containing BSA (1 mg/mL), which was added as a carrier protein, pH 5, were incubated at temperatures in the range 35–50 °C, for 0, 15, 30, 60, 120, 180, 300, 480, and 1,440 min, before performing activity assays in standard conditions.

pH optimum and stability

To determine the optimum pH for enzyme activity, reactions were performed as described previously, but using different buffers (phosphate citrate buffer 40 mM, pH 2.5–3.5; sodium citrate buffer 40 mM, pH range 4–6; sodium phosphate buffer 40 mM, pH range 6.5–8). To determine the stability of the enzyme with regard to pH, before assaying the enzyme in standard conditions, the enzyme solution was incubated for 0, 15, 30, 60, 120, 180, 300, 480, and 1,440 min, in different pH conditions (2.5–8) using the different buffers mentioned above.

Determination of product profiles

To investigate the nature of the products generated by *XynA* acting on cellodextrins, reactions were performed using cellobiose (C2), cellotriose (C3), cellotetraose (C4), and cellopentaose (C5). To achieve this, *XynA* was incubated with solutions of these compounds (500 µM) in 40 mM sodium citrate buffer (pH 5), containing 1 mg/mL BSA. When C2 or C3 was used, the concentration of *XynA* was 37.5 µM, whereas this concentration was 0.375 µM when C4 or C5 was used. Reactions were started by enzyme addition, then aliquots were removed at regular intervals and immediately placed on ice. Subsequently, reactions were definitively arrested by enzyme inactivation at 95 °C for 5 min. Following centrifugation at 14,000 rpm for 10 min, the samples were diluted 10-fold in ultrapure water, filtered (Millex-GS, 0.22 µm, mixed cellulose esters, 25 mm, nonsterile; Millipore, USA) and injected onto an anion exchange Carbopac PA100 column (Dionex, Sunnyvale, CA, USA) running on a Dionex high-performance liquid chromatography (HPLC) system equipped with pulsed amperometric detection. Oligosaccharides were eluted in 50 mM NaOH using a linear gradient of sodium acetate (from 50 to 300 mM). Glucose, C2, C3, C4, and C5 were used as standards.

Determination of IC₅₀ of cellobioses

To investigate inhibition of *XynA* by C2, hydrolysis of *pNP-β-D-Lac* by *XynA* in the presence of different concentrations (0, 10, 20, 200, and 500 μM) of C2 was performed. Reactions were conducted at 40 °C in 40 mM sodium citrate buffer, pH 5 containing 1 mg/mL BSA and 5 mM *pNP-β-D-Lac*. Release of *pNP* was monitored in a discontinuous manner by removing samples (40 μL) at regular intervals. The enzyme reaction was stopped by adding 200 μL 1 M sodium carbonate (pH 9), and the absorbance at 402 nm was measured using a Sunrise microplate spectrophotometer (TECAN, Männedorf, Switzerland). The amount of free *pNP* was determined by comparing values against a standard curve prepared using a known range of *pNP* concentrations. Afterward, the data were analyzed using a four-parameter logistic equation in SigmaPlot software (version 11.0) and the IC₅₀ value (50 % inhibition concentration) was calculated. To estimate the *K_i* value, the Cheng–Prusoff equation was used:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}}$$

Results and discussion

In this study we set out to determine the exact specificity of *XynA*, a component of the secretome of *P. funiculosus* that has previously been described as a xylanase [12]. This is because *XynA* is classified in the GH7 family in the CAZy database and a BlastP search of the Protein Data Bank (PDB), using the sequence of *XynA* (Uniprot Q8WZJ4) as the query, reveals that the catalytic domain of *XynA* exhibits high similarity to that of family GH7 CBHs from *T. emersonii* (Uniprot Q8TFL9) and *T. reesei* (Uniprot P62694.1) (72 and 65 % identity, respectively). Moreover, the presence in *XynA* of a family 1 carbohydrate binding module (CBM) is consistent with the fact that this enzyme should possess CBH activity.

To provide unequivocal biochemical data, our initial intention was to clone and express the gene encoding *XynA*, but this proved to be impossible despite the fact that several expression hosts (bacteria and yeast) were tested. Therefore, to surmount this problem, we decided to devise a robust purification method that would yield a highly pure sample of *XynA*. Accordingly, a three-step purification method involving two HIC steps was implemented to purify *XynA* from Rovabio Excel™, mainly because *XynA* was not retained on the anion exchange column (second step), although this step was useful to remove some contaminating protein species. After the third step, *XynA* was obtained in a homogeneous, pure form that migrated to a

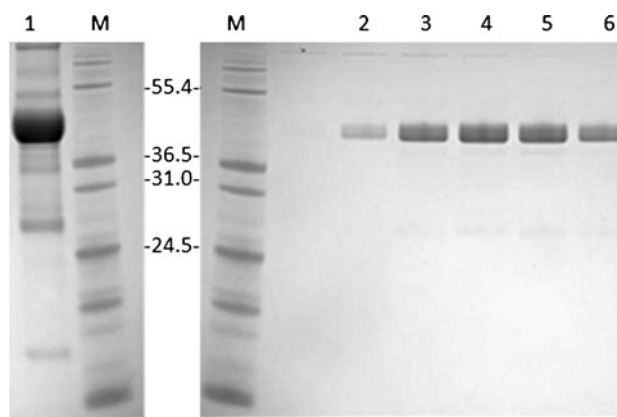


Fig. 1 SDS-PAGE analysis of the protein sample before purification (lane 1) and of the different fractions obtained using hydrophobic interaction chromatography (lanes 2–6). M is a protein molecular weight marker

position on Coomassie blue-stained SDS-PAGE (Fig. 1), corresponding to molecular mass of 52.5 kDa, which is the theoretical molecular weight for *XynA*.

To confirm that the majority protein species in the purified protein sample was *XynA* we performed protein sequence analysis, which generated sequence fragments that matched with the sequence of *XynA* (Uniprot Q8WZJ4), with fragments covering 34 % of the full protein sequence and a score of 19,692, compared with 91 for the sequence of the GH7 CBHA of *Aspergillus niger* (Uniprot Q9UVS9). This rather conclusive result confirmed that the purified species was *XynA* and thus allowed us to continue with the detailed analysis of the biochemical properties of the protein.

To begin the characterization of *XynA*, it was first necessary to determine which substrate would be most appropriate for further analyses. Therefore, activity screening was performed using substrates that would reveal both glucanase and xylanase activities. These experiments revealed that activity on MVβG was 100 times higher than the activities measured on BX (0.14 nmole equivalent xylose min⁻¹ mg⁻¹ of *XynA*) and WAX. These results contrast sharply with those published by Furniss et al. [12], which revealed activity of 45 μmol equivalent xylose min⁻¹ mg⁻¹ of *XynA* on BX.

Having ascertained that MVβG is an appropriate substrate for *XynA*, it was possible to investigate some of the key physicochemical properties of *XynA*. The determination of thermoactivity and thermostability revealed that *XynA* is optimally active at approximately 55 °C (Fig. 2a), but at this temperature its thermostability would be low, since our data suggest that at 50 °C the enzyme's half-life is less than 2.5 h (Fig. 2b). Likewise the determination of the optimum pH for activity indicated that *XynA* maintained >80 % activity on MVβG in the pH range 3–4.5

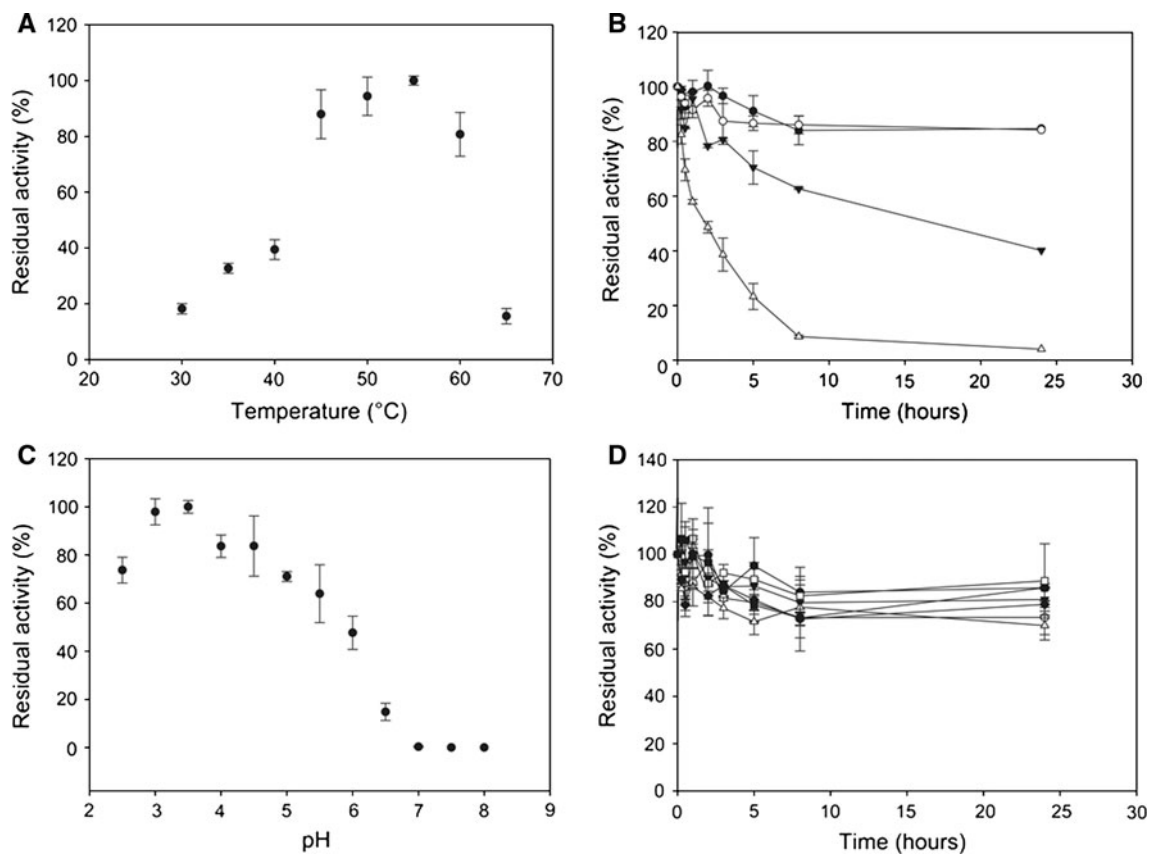


Fig. 2 Physicochemical properties of *XynA*. **a** Temperature dependency of *XynA* activity measured on medium-viscosity barley β -glucan. **b** Residual activity of *XynA* after incubation at 35 °C (filled circles), 40 °C (circles), 45 °C (filled inverted triangles), and 50 °C (triangles). **c** pH dependency of *XynA* activity measured on medium-

viscosity barley β -glucan. **d** Residual activity of *XynA* after incubation at pH 2.5 (filled circles), pH 3 (circles), pH 3.5 (filled inverted triangles), pH 4 (triangles), pH 4.5 (filled squares), pH 5 (squares), and pH 5.5 (filled diamonds)

(Fig. 2c), which agrees reasonably well with the optimum pH range (3.7–5.2) reported by Furniss et al. [12]. Indeed, even after long periods of exposure (25 h) to acidic conditions (pH 2.5–5.5), the enzyme retained approximately 80 % of its original activity (Fig. 2d). Generally, the properties described here for *XynA* are highly similar to those of many other CBH enzymes from microbial sources; For example, CBH I from *T. reesei* displays optimal activity at approximately pH 4.8 and 50 °C [21].

Regarding the kinetic parameters that were determined for the hydrolysis of the two β -glucans tested, the K_M value for the hydrolysis of MV β G was markedly lower than that of the reaction involving CMC, and the catalytic constant for the former reaction was 18-fold higher, which is perfectly coherent with a cellobiohydrolase-like exoglucanase activity, rather than that of an endoglucanase (Table 1). Similarly, the use of the synthetic glycosides *p*NP- β -D-Lac and *p*NP-cellobiose clearly revealed that *XynA* is capable of exo-acting activity, and significantly, the catalytic constant for hydrolysis of *p*NP-cellobiose was 5.2-fold higher than for *p*NP- β -D-Lac, thus revealing clear CBH I

character. It is also noteworthy that the K_M value displayed by *XynA* for hydrolysis of *p*NP-cellobiose (0.238 mM) is quite similar to those of other CBHs whose K_M values are often in the range 0.1–0.8 mM [4, 18, 20, 26]. Likewise, the turnover numbers measured on *p*NP-cellobiose and *p*NP- β -D-Lac were remarkably high, since the k_{cat} value measured for Cel7A of *T. reesei* or Cel7D of *Phanerochaete chrysosporium* on *p*NP- β -D-Lac was only 0.093 and 0.17 s⁻¹, respectively, and the k_{cat} value measured for Cex

Table 1 Kinetic parameters measured on MV β G, CMC, *p*NP-cellobiose, and *p*NP- β -D-Lac

Substrate	K_M (app)	k_{cat} (s ⁻¹)	k_{cat}/K_M
MV β G	5.095 mg mL ⁻¹ ± 0.002	4.6	0.90 mg ⁻¹ mL s ⁻¹
CMC	14.6 mg mL ⁻¹ ± 0.01	0.8	0.05 mg ⁻¹ mL s ⁻¹
<i>p</i> NP- β -D-Lac	0.57 mM ± 0.05	78.3	0.14 M ⁻¹ s ⁻¹
<i>p</i> NP-cellobiose	0.238 mM ± 0.02	174.2	0.73 M ⁻¹ s ⁻¹

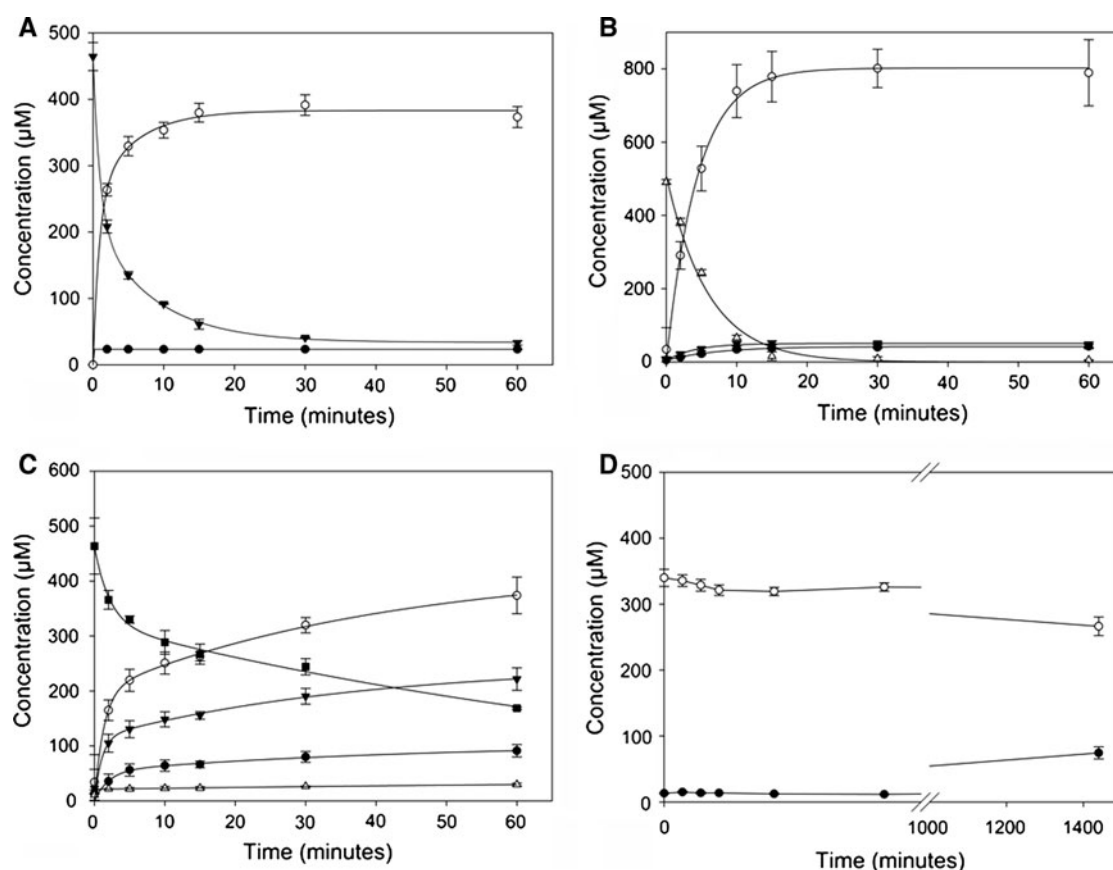


Fig. 3 *XynA*-mediated hydrolysis of cellotriose (a), cellotetraose (b), cellopentaose (c), and cellobiose (d). Symbols are cellotriose (filled inverted triangles), cellobiose (circles), glucose (filled circles), cellotetraose (filled triangles), and cellopentaose (filled squares)

of *Cellulomonas fimi* on *pNP*-cellobiose was 15.8 s^{-1} [24, 25].

Using different cellodextrins containing two to five glucosyl units as substrates revealed that *XynA* was active on all of these, although cellobiose was a poor substrate, being very slowly hydrolyzed to glucose (Fig. 3a–d). Indeed, after 24 h, the concentration of cellobiose had only diminished from 350 to 260 μM (Fig. 3d). In contrast, C3 was rapidly hydrolyzed to C2 and glucose, with the reaction reaching near completion after only 10 min (Fig. 3a). A highly similar, rapid reaction was observed for C4, with mainly C2 being produced, accompanied by only small amounts of glucose and C3 (Fig. 3b). Finally, it is noteworthy that C2 was also the major product of C5 hydrolysis, though C3 and glucose are produced, indicating that the initial hydrolysis of C5 produces C2 and C3, and that subsequent hydrolysis of C3 produces C2 and glucose (Fig. 3c). Overall, this behavior is similar to that described for Cel7A of *T. reesei*, although this latter does not release any trace of glucose when hydrolyzing cellodextrins [14]. Interestingly, in the case of C5, the use of higher concentrations of substrate $>50 \mu\text{M}$ revealed an increasingly obvious inhibitory effect, which became severe when

concentrations $>100 \mu\text{M}$ were used. Since it is well known that CBHs are often inhibited by cellobiose [17], hydrolysis of *pNP*- β -D-Lac in the presence of increasing concentrations of cellobiose was investigated. This analysis provided an IC_{50} (C2) value of 17.7 μM . Likewise, using the Cheng–Prusoff equation, it was possible to generate a K_i value of 1.8 μM [7]. Overall, these results are consistent with the observed inhibition of *XynA*, and the IC_{50} value measured for the *T. reesei* CBH I (approximately 35.3 μM), using MeUmbG2 as the substrate [32]. Nevertheless, the estimated value of K_i is lower than that observed for the *P. chrysosporium* CBH I ($K_{i \text{ C2}} = 65 \mu\text{M}$) acting on *pNP*-cellobiose [19]. Considering that some CBH1 enzymes have been reported to operate normally in the presence of C2 up to concentrations of 0.5–2 mM, it is possible conclude that *XynA* is quite sensitive to inhibition [27, 29].

Conclusions

In this study we set out to provide new biochemical data for *XynA* of *P. funiculosus*, an enzyme that is a member of the CAZy family GH7 and that has been previously described

as a xylanase, despite the fact that no other xylanases have so far been identified in family GH7. In total contrast to the data presented by Furniss et al. [12], we failed to detect any significant xylanase activity, but instead clearly measured activities on β -D-glucans and cellodextrins, with catalytic behavior that corresponds to that of a type 1 cellobiohydrolase or CBH I. Therefore, based on our data, we propose that the description of *XynA* contained within the CAZY database and elsewhere should be amended and that the enzyme should be renamed Cel7A. Beyond these recommendations, we note that, based on our data and on those already published by Guais et al. [13], *P. funiculosum* is equipped with a full arsenal of cellulose-degrading enzymes and could thus be an interesting candidate for the production of a lignocellulosic biomass-degrading enzyme cocktail.

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