Curr Microbiol (2008) 57:39–44
DOI 10.1007/s00284-008-9149-1

A Bifunctional Endoglucanase/Endoxylanase from *Cellulomonas flavigena* with Potential Use in Industrial Processes at Different pH

Odilia Pérez-Avalos · Leticia M. Sánchez-Herrera · Luis M. Salgado · Teresa Ponce-Noyola

Received: 10 January 2007 / Accepted: 7 June 2007 / Published online: 1 April 2008

Abstract *Cellulomonas flavigena* CDBB-531 was found to secrete a bifunctional cellulase/xylanase with a molecular mass of 49 kDa and pI 4.3. This enzyme was active on Remazol brilliant blue–carboxymethylcellulose (RBB-CMC) and Remazol brilliant blue–xylan (RBB-X). Based on thin-layer chromatographic analysis of the degradation products, the cellulase activity produced glucose, cellobiose, cellotriose, and cellotetraose from CMC as the substrate. When xylan from birchwood was used, end products were xylose, arabinose, and xylobiose. The bifunctional enzyme showed a pH optimum of 6 for cellulase activity and 9 for xylanase activity, which pointed out that this enzyme had separate sites for each activity. In both cases, the apparent optimum temperature was 50°C. The predicted amino acid sequence of purified protein showed similarity with the catalytic domain of several glycosyl hydrolases of family 10.

Introduction

Cellulases and xylanases, whose main function is to hydrolyze β-1,4-glycosidic linkages in the major plant structural polysaccharides, are widely distributed in nature [5]. These enzymes have been used in several industries, including the food, brewery, wine, textile, pulp, and paper industries [1]. Fungi and bacteria are the most common and useful organisms used to isolate the enzymes because they synthesize a considerable number of enzymes. However, bacterial cellulases and xylanases have a neutral to alkaline optimum pH, which make them more useful in several industrial processes.

Cellulomonas flavigena is able to grow on several agricultural wastes. It produces different cellulolytic and xylanolytic complexes according to the availability of the carbon source [14]. This multiplicity of enzyme activities appears to be the result of three major factors: the existence of multigene cellulase families, glycosylation of cellulase gene products, and cleavage of cellulase gene products by host-produced proteolysis [18]. However, the role of individual cellulases and xylanases in the mixture of enzyme activities has not been clearly determined. The isolation and purification of individual components of cellulolytic and xylanolytic enzymes could provide information about their relative importance in the enzymatic hydrolysis of lignocellulose. In the present study, an extracellular protein from *C. flavigena* with activity over cellulose and xylan was purified from cultures grown on sugarcane bagasse. Other biochemical properties of this enzyme were also studied. The amino acid sequence of purified protein revealed that this is a bifunctional glycosyl hydrolase from family 10.

Materials and Methods

Microorganisms and Culture Conditions

Cellulomonas flavigena CDBB531 was grown as reported previously [11]. Cells grown on glucose were used to isolate genomic DNA. *Escherichia coli* DH5α was used as the

O. Pérez-Avalos · L. M. Sánchez-Herrera · T. Ponce-Noyola (✉)
Departamento de Biotecnologia y Bioingenieria, CINVESTAV, Av. IPN # 2508 Col. Zacatenco, C.P. 07000 D.F., Mexico
e-mail: tponce@cinvestav.mx

L. M. Salgado
Departamento de Bioquimica, CINVESTAV, Av. IPN # 2508 Col. Zacatenco, C.P. 07000 D.F., Mexico

transforming host, and *E. coli* XLI-Blue MRA (P2) (Stratagene) was used as the phage λ Fix II host. All *E. coli* strains were grown at 37°C on Luria-Bertoni (LB) broth supplemented with ampicillin (0.1 mg/mL), maltose (2 g/L), and $MgSO_4$ (10 mM).

Production and Purification of Enzyme

Cellulomonas flavigena was grown for 48 h as described earlier in a Fernbach culture flask containing 1 L of culture medium. The residual substrate was removed by filtration through a GD120 glass fiber filter disk (MFS, Dublin, CA). Cells were recovered by centrifugation at 5000*g* for 10 min at 4°C and the supernatant was used for the next purification steps. Culture supernatant was concentrated 10-fold through a PM-10 membrane (Amicon, Beverly, MA, USA). The concentrated crude extract was mixed with acetone (3:1) and the precipitate was recovered by centrifugation. The pellet was resuspended in 25 mM Tris-HCl (pH 7.2) and applied to a Bio-Gel P100 gel filtration column (1.5 × 20 cm), equilibrated with the same buffer at a flow rate of 0.1 mL/min. The fractions with cellulolytic activity were pooled, lyophilized, and dissolved in 1 mL of 25 mM Tris-HCl (pH 7.2). This sample was loaded onto a hydroxylapatite column (1.5 × 20 cm) (Bio-Rad), equilibrated with 50 mM potassium phosphate buffer (pH 6.8). The column was eluted with 200 mM KCl, in the same buffer, at a flow rate of 0.25 mL/min. Fractions with high cellulase activity were lyophilized and loaded again onto the hydroxylapatite column. The purified enzyme was used for further characterization. The purification process was carried out at 4°C.

Enzyme Activity Assays

Cellulase and xylanase activities were determined by measuring the released cellobiose and xylose, respectively, as previously reported [15]. An international unit of activity (IU) is defined as the amount (micromoles) of cellobiose or xylose released per minute under standard assay conditions. All determinations were performed in three independent experiments.

Determination of Protein

Protein concentration was measured by the Bradford method [3] or by monitoring ultraviolet (UV) absorbance at 280 nm.

Electrophoretic Methods

Enzyme purity was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [9]. Bands were visualized using Coomassie brilliant blue G250. For identification of cellulase or xylanase activities, samples of 25 μg of protein were subjected to PAGE containing 0.1% Remazol brilliant blue–carboxymethylcellulose (RBB-CMC) or 0.1% RBB-X (xylan) prepared according to Biely et al. [2]. The gels were incubated at 50°C for 2 h or until clear zones were detected. Xylanase activity was determined in citrate phosphate buffer (pH 7) and cellulase activity was determined in Tris-HCl (pH 7.2). Isoelectric focusing was carried out in 5% polyacrylamide gels using a Mini IEF cell apparatus (Bio-Rad). The pH gradient was created using Ampholine, pH 3.5–10 (Sigma). Focusing was carried out at 200 V for 50 min. Protein bands were visualized with Coomassie brilliant blue G250 (Sigma) or by the silver staining (Bio-Rad) method. Proteins of known pI, 3.5–9.3, were used as standards (Sigma).

Temperature and pH Optima

Optimum pH was estimated using the cellulase or xylanase activity assay over a pH range between 5 and 10: sodium citrate–phosphate buffer for pH range 5–7; phosphate buffer for pH range 5–8, and Tris-HCl buffer for pH range 7–10. Optimum apparent temperature was estimated using the cellulase or xylanase activity assay at temperatures between 30°C and 70°C.

Enzyme Kinetic Analysis

The linear region of the rate of release of cellobiose from CMC or of xylose from xylan was determined for different times and enzyme concentrations. CMC or xylan (1 mL) at several concentrations (0.25–20 mg/mL) was incubated as indicated previously and the kinetic constant K_m and V_{max} were estimated using a Lineweaver–Burk plot. All assays were performed in triplicate.

End-Product Determination

Purified enzyme (25 μg) was incubated in an orbital water bath shaker at 50°C, 100 rpm for 96 h with 10 mg of xylan in 25 mL of sterile Tris-HCl buffer (pH 9) for xylanase activity, or 25 mg of CMC in 25 mL of sterile citrate–phosphate buffer (pH 6) for cellulase activity. Samples of 1 mL were taken at 0, 15, 24, 48, 72, and 96 h, lyophilized, and resuspended in 50 μL Tris-HCl buffer (pH 7). Aliquots of 10 μL were spotted on thin-layer chromatography (TLC) silica gel 60 plates (Merck, Germany), along with standards of glucose, cellobiose, and cellotriose for cellulose hydrolysis and arabinose, xylose, and xylobiose for xylan hydrolysis. Separation was carried out with acetic acid: 1-butanol:water (1:2:1 v/v) at room temperature. Released

sugars were detected by spraying the plates with 0.2% acidic orcinol and then heating at 100°C for 15 min [11].

Protein Sequence

The purified protein band after SDS-PAGE was sequenced according to Kinter and Sherman [8] at the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia. The peptides were matched in the database to find homology with other glucanases (cellulases and/or xylanases).

Cloning and Other Molecular Biology Techniques

The cloning vector was *pBluescript* SK II (Stratagene). The genomic DNA of *C. flavigena* and plasmid DNA were isolated according to standard protocols [16]. Primers, forward (acsgcsgagaacgagatg) and reverse (scgsacgaactgvccsgactg), were designed according to the sequence of the peptides EFNMX**TAENEM**K and **SQSGQFV**R, respectively. The conditions for the PCR reactions were as follows: 60 ng genomic DNA, 12 pmol primers, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1X buffer, and 2.5 units of *Taq* DNA polymerase (Invitrogen) in a final volume of 50 µL. The reaction conditions were hot-started at 94 °C for 5 min, one cycle; followed by 30 cycles of 1 min at 94°C, 1 min at 49°C, and 1 min at 72°C, in a Gen Cycler (Bio-Rad). The PCR product (387 bp) was purified from 1% agarose gels with the QIAEXII Gel Extraction Kit (Qiagen) and cloned in the *Eco*RV site of p*Bluescript* SK II. The cloned product was sequenced using the Reading Reaction Cycle Sequencing Kit (Perkin-Elmer) in an ABI 373 DNA sequencer (Applied Biosystems Inc.). The sequence analysis software BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and Workbench 3.1 (http://www.workbench.sdsc.edu) were used for the alignment of the sequences.

Nucleotide Sequence Accession Number

The *cxo* nucleotide sequence data has been submitted to the GenBank database under accession No. DQ441404.

Results

Enzyme Purification

Cellulomonas flavigena produces several extracellular cellulases and xylanases when it is grown in cellulosic substrates as the sole carbon source. In this way, after several purification steps, a protein comprising cellulase and xylanase activities was purified to apparent homogeneity through Bio-Gel P100 and hydroxylapatite columns. Only one peak with cellulase activity was eluted from a hydroxylapatite column with 200 mM KCl. This fraction displayed only one protein band when was run in SDS-PAGE with an apparent molecular weight of 49 kDa. This observation confirmed the purification of the protein to homogeneity. After the final step, the 49-kDa cellulase from *C. flavigena* was purified 39-fold with 11% activity yield (Table 1).

The Purified Enzyme Comprises Two Activities

The purified enzyme showed only one band in SDS-PAGE (Fig. 1a) and in PAGE (Fig. 1b). However, the zymograms showed independent CMCase and xylanase activities (Fig. 1c). This result points out that this enzyme is bifunctional and that it is capable of hydrolyzing both β-1,4-glycosidic and β-1,4-xylosidic bonds in cellulose and xylan, respectively.

Biochemical Properties

According to the above results, the purified enzyme was assayed for cellulase and xylanase activities at different pHs. The highest cellulase activity was observed in citrate–phosphate buffer at pH 6 and the best activity in phosphate buffer was at pH 7, ~10% less than in citrate–phosphate buffer. When a higher pH was used to measure the CMCase activity, in Tris-HCl the activity was diminished to less than 50% of the highest obtained at pH 6. For the xylanase, the highest activity was observed at pH 9 in Tris-HCl buffer, whereas in citrate–phosphate buffer, pH 7, the best activity was 90% of that observed in Tris-HCl. The

Table 1 Purification of endoglucanase/endoxylanase of *C. flavigena*

Purification step	Total protein (mg)	Cellulase activity (IU/mg)	Yield (%)	Purification (X-fold)
Crude extract	34.00	6.7	100	1.0
Amicon PM-10	17.00	8.8	65	1.3
Biogel P100	3.00	25.0	32	3.6
Hydroxylapatite	0.19	210.0	17	31.0
Hydroxylapatite	0.10	263.0	11	39.0

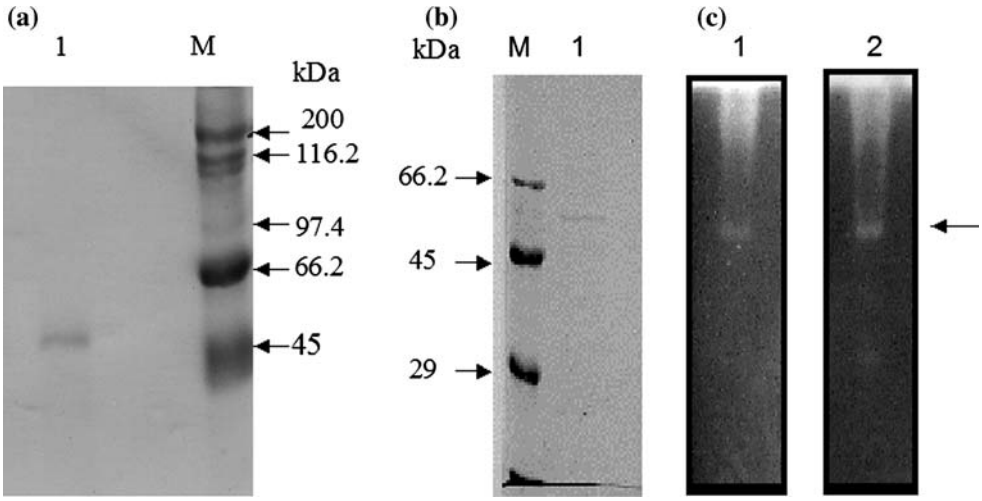

Fig. 1 PAGE of the protein eluted from a hydroxylapatite column: (**a**) protein separated under denaturing conditions, from *C. flavigena*, stained with Coomassie brilliant blue. M, molecular weight standards; (**b**) protein separated under native conditions and stained with Coomassie blue (lane 1); (**c**) zymogram using RBB-CMC (lane 1) or RBB-xylan as the substrate (lane 2)

phosphate buffer had a negative effect on the xylanase activity, as it reduced the activity around to 60%. The endoglucanase/endoxylanase from *C. flavigena* was found to be an acidic enzyme with a pI of 4.3. The optimum temperature, for both cellulase and xylanase activities, was 50°C. The Arrhenius law was followed between 30°C and 50°C and the activation energy for the cellulase and xylanase activities was 8498 and 9258 cal/mol, respectively, calculated with a Q_{10} of 0.75. Under the experimental conditions, the rate of release of xylose or cellobiose, from xylan or CMC, respectively, was linear for 5 min and 0.5 mg protein/mL. The apparent K_m and V_{max} values for cellulase and xylanase activities were 0.16 and 0.72 mg/mL and 971 and 2854 IU/mg, respectively, according to Lineweaver–Burk plots (Table 2).

Detection of Hydrolysis Products

Glucose, cellobiose, and cellotriose were found as the hydrolysis products of CMC (Fig. 2a). When the xylanase activity was examined, xylose, arabinose, and xylobiose from xylan were detected (Fig. 2b). In both cases, high-molecular-mass oligosaccharides were also observed.

Sequence Analysis of the Endoglucanase/Endoxylanase Protein

The single 49-kDa-protein band was subjected to proteolysis with trypsin and partially sequenced using liquid chromatography–mass spectrometry. The sequence of 15 peptides was obtained and some showed identity with

Table 2 Kinetic parameters of endoglucanase/endoxylanase of *C. flavigena*

Enzyme activity	Optimum pH[a]			Optimum T (°C)	K_m (mg/mL)	V_{max} (IU/mg)
	a	b	c			
CMCase	6.0	7	–	50	0.16	971
Xylanase	7.0	–	9.0	50	0.72	2854

[a] a = Citrate–phosphate buffer, b = phosphate buffer, c = Tris-HCl buffer

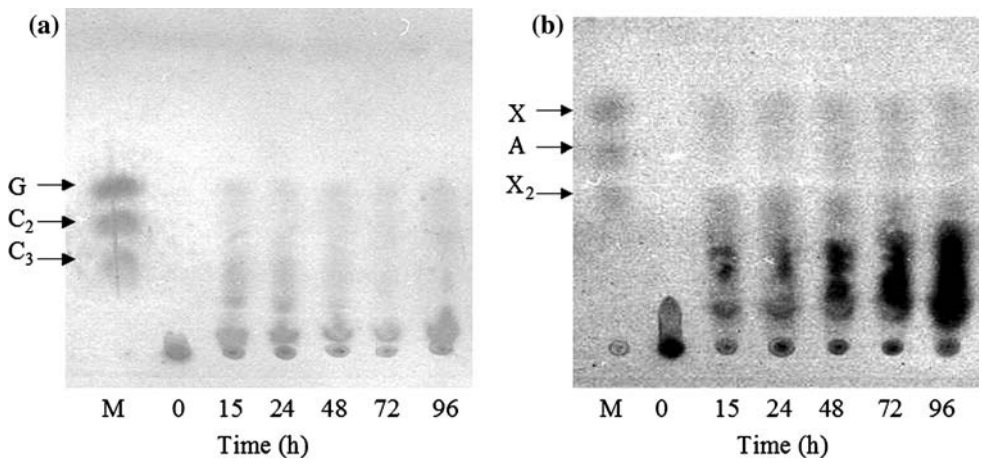

Fig. 2 Time course of end products from (**a**) CMC or (**b**) xylan hydrolysis for purified protein from *C. flavigena*. G = glucose, C_2 = cellobiose, C_3 = cellotriose, X = xylose, A = arabinose, X_2 = xylobiose

amino acid sequences from published cellobiohydrolases and xylanases of family 10 of glycosyl hydrolases.

Two peptides were chosen to design oligonucleotides to amplify a polymerase chain reaction (PCR) product from genomic DNA. A single band of 387 bp was obtained, which was cloned and sequenced. The analysis of the sequence showed that this fragment could be part of the catalytic domain of the endocellulase/endoxylanase purified from *C. flavigena*. The amino acid sequence was compared against the sequences reported in the GenBank and significant identity was observed with the catalytic domain of bifunctional cellulases and xylanases (data not shown). The nucleotide sequence defines part of the gene named *cxo* from *C. flavigena*, which codes for the bifunctional enzyme of 49 kDa.

Discussion

Cellulomonas flavigena has several properties that make it a suitable organism for biotechnological purposes. Based on the distinct approaches that we have used, traditional biochemical methods give good results, even though other approaches have been used as well [6, 11, 12]. From our biochemical screening of cellulolytic enzymes, we purified a new protein with two catalytic activities. The protein of 49 kDa secreted by *C. flavigena* was able to hydrolyze two substrates, CMC and xylan, producing the expected oligosaccharides and disaccharides from them. The biochemical properties of the protein and the end products obtained from the two substrates provide strong evidence that the 49-kDa protein is a bifunctional enzyme with endotype activities and this protein is an endoglucanase/endoxylanase.

The fact that the optimum pH for the two activities of the protein was different convincingly suggests that this protein has two active sites: one for each activity. Similar behavior was observed in the xylanase *Xyn*D from *Ruminococcus flavefaciens* [4]. This acid/base characteristic of the endoglucanase/endoxylanase of *C. flavigena* could be attributed to the presence of glutamic acid in the catalytic domain, which allows for this biochemical property. Glu is part of the highly conserved region WDVVNEA found in one of the peptides obtained from the partial sequence of the 49-kDa protein of *C. flavigena.* This sequence has an important function in the structure of the catalytic domain of a thermostable xylanase of *Thermoascus aurantiacus* [13], whereas Glu 127 gives the acid/base catalytic function in the catalytic domain of Cex of *C. fimi* [10].

The alignment of the partial amino acid sequence and the nucleotide sequence of the 387-bp band obtained from the PCR product matched with several xylanases. The identity varied from 48% against the Cex exoglucanase of *C. fimi* (Q59277) to 73% of the xylanase A of *Streptomyces coelicolor* (T35696). Enzymes of this type are classified in the family 10 of the glycosyl hydrolases consisting of nonspecific glucanases that are able to hydrolyze xylan and numerous glucose-derived substrates such as cellulose [7]. *In vitro*, the members of family 10 act on both para nitro phenol (PNP)-xylobiose and PNP-cellobiose. However, the overall catalytic efficiency with PNP-xylobioside as the substrate is about 50 times higher, suggesting that these enzymes act mainly on xylan [17].

It is important to consider the dual activity of the endocellulase/endoxylanase from *C. flavigena*, which can be used in saccharification of agricultural, industrial, and municipal wastes to provide sugar syrups for animal feed or in the production of fuel chemicals through industrial fermentations. In the pretreatment of paper pulps, removing xylan while preserving cellulose content, such as at pH 9, the xylanase activity would be high and the cellulase activity would be very low according to the biochemical properties of the bifunctional enzyme. Likewise, in a process that needs the cellulolytic activity under acid conditions (i.e., pH 6), the xylanolytic activity will be maintained low.

Acknowledgments This work was supported by Consejo Nacional de Ciencia y Tecnología-México (45678-Z). The authors thank the W. M. Keck Biomedical Mass Spectrometry Laboratory UVHS for sequencing the protein and Martha Mercado-Morales for technical assistance.

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