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Comparison of properties and mode of action of six secreted xylanases from *Chrysosporium lucknowense*

Boris B. Ustinov, Alexander V. Gusakov *, Alexey I. Antonov, Arkady P. Sinitsyn

Division of Chemical Enzymology, Department of Chemistry, M. V. Lomonosov Moscow State University, Moscow 119992, Russia

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Abstract

Eight homogeneous xylanases were purified from crude multienzyme preparations produced by various mutant strains of the fungus *Chrysosporium lucknowense*. Peptide mass fingerprinting showed that the isolated enzymes are the products of six different genes of *C. lucknowense*, three of them encoding xylanases belonging to family 10 of glycoside hydrolases (GH) and three other genes encoding enzymes of the GH11 family. Intact Xyn10A and Xyn10B possessed a family 1 CBM at the N- and C-terminus, respectively; each of the enzymes was also isolated in the form without CBM. The GH11 family xylanases displayed very high specific activities against various xylans, the Xyn11A being the most active (329–494 U mg^{-1}). In hydrolysis of glucuronoxylan and arabinoxylan, xylanases belonging to the same family were characterized by very similar kinetic behavior and composition of the final products. The GH10 family xylanases showed greater catalytic versatility and formed shorter oligosaccharides than those of family 11. Xyn10A, Xyn10B and Xyn11A were characterized by broad pH optima and displayed high activity in neutral and moderate alkaline medium. The GH10 family xylanases demonstrated high thermostability retaining more than 70% of activity after 1-h incubation at 60 °C. These properties make the *C. lucknowense* xylanases promising candidates for different biotechnological applications.

Keywords: *Chrysosporium lucknowense*; Glycoside hydrolases; Hemicellulose degradation; MALDI-TOF mass spectrometry; *Thermomyces lanuginosus*; Xylanase

1. Introduction

Xylanases (endo-1,4-β-xylanases, EC 3.2.1.8) are enzymes hydrolyzing β-1,4-glycosidic linkages in the backbone of xylans, the major component of hemicelluloses in the plant cell wall [1–3]. Xylanases are widely abundant in nature; they are produced by bacteria, fungi, protozoa, algae, gastropods, arthropods, nematodes, etc. [3,4]. Microbial xylanases are the most studied enzymes compared to those from other organisms. Most of known fungal xylanases belong to families 10 and 11 of glycoside hydrolases (GH), according to modern classification introduced by Henrissat et al. [5,6] that is based on amino-acid sequence similarities. Xylanases from bacteria and other organisms may also be found in the GH families 5, 8, 16, 43, 62 (http://www.cazy.org/).

Microbial xylanases found different biotechnological applications in the last two decades. They are widely used in the process of cellulose biobleaching in the pulp and paper industry to reduce the usage of chlorine, in the food industry (brewing, bakery, fruit and vegetable processing, juice and wine making, etc.), and as feed additives in broiler and animal diets [2–4,7]. In the nearest future, the potential large-scale application of xylanases (together with cellulases) may become the bioconversion of plant biomass to ethanol and other useful products [2,7–9]. Other potential xylanase applications may be realized in textile and pharmaceutical industries [3,4].

The growing fields of xylanase applications require searching for novel enzymes and new microbial producers with higher specific activities, higher productivity and other important properties. Xylanases that are active at alkaline pH and/or with enhanced thermostability are often required in some biotechnological processes [2,3,7]. So, the task of finding new xylanases with necessary properties remains topical.

Chrysosporium lucknowense is filamentous fungus that produces a wide range of enzymes that catalyse the biodegradation of cellulose and hemicelluloses. Industrial mutant strains of *C. lucknowense*, characterized by extremely high productivity and low viscosity of the fermentation medium, have been developed recently [10,11]. The sequencing of the full fungal genome of the *C. lucknowense* followed by the genome annotation

* Corresponding author. Tel.: +7 495 939 5966; fax: +7 495 939 0997.
E-mail address: avgusakov@enzyme.chem.msu.ru (A.V. Gusakov).

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(http://www.dyadic.com/wt/dyad/pr_1143820822) allowed discriminating about 200 genes encoding carbohydrate-active enzymes that may be potentially interesting from the point of view of biotechnological applications. However, literature data on enzymes from the fungi belonging to genus *Chrysosporium* are very scarce. Previously, we have isolated and studied properties of a few cellulases from *C. lucknowense*; the endo-1,4-β-glucanase II (Cel5A), cellobiohydrolases Ia (Cel7A) and IIb (Cel6B) distinguished from other cellulases by remarkably high specific activity and/or thermostability [12–14]. Based on the three mentioned enzymes, artificial multienzyme mixtures were reconstituted, displaying an extremely high performance in hydrolysis of different cellulosic substrates [14]. Xyloglucan-specific exo-β-1,4-glucanase (Xgl74A) is another interesting enzyme found in *C. lucknowense* [15]; because of its unique substrate specificity the enzyme was given a new number in the Enzyme Nomenclature (EC 3.2.1.155).

Eleven genes encoding different xylanases were recently found in the *C. lucknowense* genome. However, some of them seem to represent silent or low-productive genes with low expression of the corresponding proteins. In this paper, we describe the properties and mode of action of *C. lucknowense* xylanases encoded by six different genes. Since two enzymes existed in the fermentation broths in different forms, eight xylanases in total were purified and characterized.

2. Materials and methods

2.1. Enzymes

Crude multienzyme preparations NCE L-600, CX1-56, C1-1170 and CT1-13 produced by various mutant strains of *C. lucknowense* (from Dyadic International Inc., USA) were used for isolation of xylanases. The first preparation mentioned was a Dyadic commercial product, while other preparations represented laboratory samples with enhanced xylanase content. Homogeneous α-L-arabinofuranosidase (70 kDa) was isolated from *Penicillium canescens* PCA10 strain as described previously [16].

2.2. Purification of xylanases

Purification of *C. lucknowense* xylanases was carried out by chromatography on a Pharmacia FPLC system (Sweden). The first stage was anion-exchange chromatography on a Source 15Q column (3.6 cm × 3.0 cm). The column was equilibrated with 25 mM bis-Tris/HCl buffer, pH 6.6. The initial enzyme preparations were desalted and transferred into the starting buffer by gel-filtration on Acrylex P4 (Reanal, Hungary). The samples (200–700 mg of protein) were applied to the Source 15Q column, and the elution was carried out with a gradient of 0–0.35 M NaCl in equilibration buffer at a flow rate of 10 ml min^{-1}. Fractions containing xylanase activity were subjected to further purification using different types of chromatography.

The non-bound protein fraction after chromatography of the NCE L-600 preparation on Source 15Q was transferred on a Mono Q HR 10/10 column (1 cm × 10 cm) equilibrated with 25 mM Tris/HCl buffer, pH 7.8. The elution was carried out with a gradient of 0–0.2 M NaCl at a flow rate of 2 ml min^{-1}. Fractions containing xylanase activity were subjected to hydrophobic-interaction chromatography on a Source 15 Isopropyl HR 5/5 column (0.5 cm × 5 cm) in 50 mM Na-acetate buffer, pH 5.0. Proteins were eluted with a reversed linear gradient of 1.7–0 M ammonium sulfate at a flow rate of 1 ml min^{-1}. As a result, different forms of Xyn10A and Xyn10B were obtained as homogeneous enzymes.

The non-bound protein fractions after chromatography of the CX1-56 and C1-1170 preparations on Source 15Q were subjected to further purification on a Source 15 Isopropyl column as described above. Homogeneous Xyn11B (from CX1-56) was obtained as a result of the hydrophobic-interaction chromatography. Other xylanase fractions after Source 15 Isopropyl were concentrated on a Phenyl Superose HR 5/5 column (0.5 cm × 5 cm) and further purified by gel-filtration on a Superose 12 column (1 cm × 30 cm) in 0.1 M Na-acetate buffer, pH 5.0. Homogeneous Xyn11A (from CX1-56) and Xyn11C (from C1-1170) were obtained as a result of these purification steps.

Xylanase fraction from the CT1-13 preparation, eluted from the Source 15Q column at ~0.15 M of NaCl, was further purified on a Source 15 Isopropyl column as described above. Homogeneous Xyn10C was found in the first protein fraction after the hydrophobic-interaction chromatography.

The enzyme purity was characterized by SDS-PAGE and isoelectrofocusing. SDS-PAGE was carried out in 12% gel using a Mini Protean II equipment (Bio-Rad Laboratories, USA). Isoelectrofocusing was performed on a Model 111 Mini IEF Cell (Bio-Rad Laboratories, USA). Staining of protein was carried out with Coomassie Blue.

2.3. MALDI-TOF mass-spectrometry

The in-gel tryptic digestion of the protein bands after the SDS-PAGE was carried out essentially as described by Smith [17]. Sequencing grade modified trypsin (Promega, USA, 5 μg ml^{-1}) in 50 mM ammonium bicarbonate was used for a protein digestion. The resulting peptides were extracted from a gel with 20% aqueous acetonitrile containing 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectrometry (MS) using α-cyano-4-hydroxycinnamic acid as a matrix [18]. The MALDI-TOF MS of proteins was carried out using sinapinic acid as a matrix. Autoflex II mass spectrometer (Bruker Daltonik Gmbh, Germany) was used in the MS experiments.

2.4. Enzyme activity assays

Xylanase activity was determined by analysing reducing sugars released after 10 min of enzymatic reaction with 5 mg ml^{-1} xylan at pH 5.0 (0.05 M Na-acetate buffer) and 50 °C as described elsewhere [16]. Wheat arabinoxylan (Megazyme, Australia), arabinoxylan from oat spelts, birch and beechwood glucuronoxylans (Sigma, USA) were used as substrates in xylanase activity assays. Cellulase activity was measured by assaying reducing sugars released after 5 min of enzymatic reaction with 5 mg ml^{-1} carboxymethylcellulose (CMC, medium viscosity, Sigma, USA) at pH 5.0 and 50 °C [13,16]. Reducing sugars were analyzed by the Somogyi–Nelson method [19]. Activities against *p*-nitrophenyl derivatives of glycosides (Sigma, USA) were determined at pH 5.0 and 40 °C as described elsewhere [13]. Protein concentration was determined by the Lowry assay [20] using bovine serum albumin as a standard. All activity assays were carried out in duplicates, the standard deviations did not exceed 7%.

All activities were expressed in International Units, that is, one unit of activity corresponded to the quantity of enzyme hydrolysing 1 μmol of substrate or releasing 1 μmol of reducing sugars (in glucose equivalents) per minute.

Study of pH effect on the enzyme activity was carried out using birchwood glucuronoxylan as a substrate. Buffer mixtures containing 0.1 M citric acid and 0.2 M Na_2HPO_4 were used for maintaining the necessary pH (2.5–8.0) in the reaction system. Carbonate buffer (0.5 M) was used for creating pH > 8.0.

The temperature optima of xylanase activity were determined by assaying the reducing sugars released from birchwood glucuronoxylan after 10 min of the enzymatic reaction at 30–90 °C and pH 5.0.

For studying xylanase thermostability, the enzymes (0.2 mg ml^{-1}) were incubated at 50 or 60 °C in 0.1 M acetate buffer (pH 5.0) and 0.1 M phosphate buffer (pH 7.0) for 1 h, and then residual xylanase activity was assayed under standard conditions (50 °C, pH 5.0) [16].

2.5. Study of kinetics and enzyme mode of action

Progress kinetics of birchwood glucuronoxylan and oat arabinoxylan hydrolysis was studied for 30 h at pH 5.0 and 50 °C using the substrate concentration of 5 mg ml^{-1}. The enzymes were equalized by the activity in the reaction system (0.01 U ml^{-1}). In the course of the reaction, 300 μl aliquots were taken, incubated in boiling water bath for 5 min to inactivate the enzyme, centrifuged and then analyzed for reducing sugars released using the bicinchoninic acid assay