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Heterologous expression, purification and characterization of three novel esterases secreted by the lignocellulolytic fungus *Penicillium purpurogenum* when grown on sugar beet pulp

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ABSTRACT

The lignocellulolytic fungus, *Penicillium purpurogenum*, grows on a variety of natural carbon sources, among them sugar beet pulp. Culture supernatants of *P. purpurogenum* grown on sugar beet pulp were partially purified and the fractions obtained analyzed for esterase activity by zymograms. The bands with activity on methyl umbelliferyl acetate were subjected to mass spectrometry to identify peptides. The peptides obtained were probed against the proteins deduced from the genome sequence of *P. purpurogenum*. Eight putative esterases thus identified were chosen for future work. Their cDNAs were expressed in *Pichia pastoris*. The supernatants of the recombinant clones were assayed for esterase activity, and five of the proteins were active against one or more substrates: methyl umbelliferyl acetate, indoxyl acetate, methyl esterified pectin and fluorescein diacetate. Three of those enzymes were purified, further characterized and subjected to a BLAST search. Based on their amino acid sequence and properties, they were identified as follows: RAE1, pectin acetyl esterase (CAZy family CE 12); FAEA, feruloyl esterase (could not be assigned to a CAZy family) and EAN, acetyl esterase (former CAZy family CE 10).

1. Introduction

Lignocellulose (wood, annual plants, agricultural waste, etc.) is the most abundant renewable material on earth, and therefore, an inexhaustible source of raw material for obtaining a variety of products of industrial use, among them biofuels [1]. The rising cost of energy, related to the progressively lower availability of oil and the increase in environmental contamination resulting from the use of fossil fuels, is stimulating the development of alternative and renewable sources of energy. One of them is the generation of biofuels (particularly bioethanol) based on the degradation (or saccharification) of the lignocellulose carbohydrate components, followed by fermentation of the products [2]. The saccharification process may be performed by the use of chemical or enzymatic methods. The latter are preferred because they are more specific and can be performed under mild and non-contaminating conditions.

Pectin is one of the polysaccharide components of lignocellulose and it is present mainly in the primary cell wall of plants [3]. In its structure, several constituents are recognized: 1) Homogalacturonan: it is a polymer of α-1,4-linked-D-galacturonic acid which may be methyl-esterified at the C-6 carboxyl or acetylated at the O-2 or O-3.2) Rhamnogalacturonan I (RG I): it has a backbone of repeating units of [α-D-galacturonic acid-1,2-α-L-rhamnose-1,4]n; it may be substituted at the C-4 position of rhamnose by arabinan, galactan, or arabinogalactan side chains and acetyl and methyl esterified at the galacturonic acid residues. 3) Rhamnogalacturonan II, composed of a short stretch of galacturonic acid and substituted by complex side-chains containing at least 12 different monosaccharides [4]. RG I may also contain feruloyl ester substitutions, and these feruloyl groups may form diferulic bridges between the galactan and/or the arabinan side-chains of rhamnogalacturonan I (RG I) [3].

In the enzymatic degradation of pectin components participate a large number of enzymes (generically known as pectinases), which specifically recognize sugars and the linkages binding them and their substituents. The main groups are glycanases

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(hydrolyzing glycosidic bonds) and esterases.

Esterases form a large group of enzymes. They catalyze the hydrolysis of esters, generating an acid and an alcohol as products. Among them are the carboxyl ester hydrolases (E.C. 3.1.1.x). Two principal groups of carboxyl ester hydrolases have been identified: lipases and "true" esterases or carboxyl esterases. While lipases act on long chain fatty acid triglycerides, carboxyl esterases use simple esters or short-chain fatty acid (4 carbons) triglycerides as substrates [5]. In addition, numerous other carboxyl ester hydrolases have been recognized and classified based on substrate specificity, and E.C. numbers up to E.C. 3.1.1.99 (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/cont3a.html).

An additional classification, which includes esterases acting on carbohydrate substrates, is found in the CAZy database (http://www.cazy.org/Carbohydrate-Esterases.html). It comprises enzymes that catalyze the de-O or de-N-acylation of substituted saccharides. This classification defines 16 carbohydrate esterases (CE) families (up to March 2017), based mainly on amino acid sequence similarities.

For a number of years, our laboratory has been interested in the lignocellulose biodegradation process utilizing as a model a locally isolated strain of the fungus *Penicillium purpurogenum* [6]. This soft-rot fungus grows on a variety of natural carbon sources (wheat straw, corncob, sugar beet pulp, etc.) [7] and it secretes to the medium a large number of glycanases and esterases acting on cellulose, xylan or pectin [8,9]. Recently, the genome sequence of the fungus has been obtained (unpublished), thus opening the possibility of mining genes and analyzing new potential lignocellulolytic enzymes.

In this work, *P. purpurogenum* has been grown on sugar beet pulp. This product is composed of 20% cellulose and 50% pectin, and it includes 1.8% methanol, 3.9% acetic acid and 1% phenolic acids (ferulate and coumarate) which are esterified to the pectin [10]. The purpose of this research has been to search for novel esterases which may be present in the culture supernatant. As a result, three new esterases have been identified, expressed heterologously, purified and characterized.

2. Materials and methods

2.1. Microbial strains utilized

The source of the enzymes was the fungus *P. purpurogenum* ATTC MYA-38 which was kept in potato-dextrose agar plates. *E. coli* strains DH5α or TOP10F′ were used for cloning. *Pichia pastoris* GS115, supplied in the EasySelect Pichia Expression Kit (Invitrogen, CA, USA), was the host for heterologous expression.

2.2. Enzyme production by *P. purpurogenum*

P. purpurogenum was grown in Mandels medium [11] with sugar beet pulp at 1% as carbon source. Liquid cultures (12 L) were incubated for 7 days at 28 °C in a shaker at 200 RPM. After filtration on cheesecloth and centrifugation, the clear supernatant was concentrated using a Minitan (Millipore, U.S.A) ultrafiltration apparatus (10 KDa cutoff membrane) and Amicon ultrafiltration Centricons (Millipore), to a final volume of about 20 mL.

2.3. Partial purification of esterases

A crude separation of esterases was achieved by chromatography of the concentrated supernatant. First, a pseudo-affinity chromatography was performed using Cibacron Blue Sepharose. A column containing 50 mL of resin was equilibrated with 50 mM citrate buffer pH 4 and 15 mL of the concentrated supernatant were added. The column was washed with 3 vol of the buffer and eluted with 3 vol of a gradient (0–1 M NaCl) in the buffer. Active fractions obtained in the washing were then applied to a column containing 8 mL DEAE Sephadex A-25 equilibrated with 50 mM Tris-HCl pH 7. After washing the column with 3 vol of buffer, the proteins were eluted with a gradient of 0–1 M NaCl in the buffer. To purify the heterologously expressed enzyme EAN (see below), DEAE Sephadex A-50 was used, following a similar procedure.

2.4. Detection of enzyme activity in zymograms

Polyacrylamide gels were prepared according to Laemmli [12]. Samples (concentrated chromatography column eluates) contained 25–100 μg protein in 60 mM Tris HCl buffer pH 6.8, 25% glycerol and 0.1% Bromophenol Blue. The samples were loaded directly on the gel and the electrophoresis was performed at 4 °C for 3 h at 80 V. The gels were then washed 3 times with 50 mM acetate buffer pH 5 and were then placed in 100 mL of the same buffer containing 1 mg methyl umbelliferyl acetate (MUA) dissolved in absolute ethanol. The appearance of fluorescence bands was photographed under UV light. The gels were then washed with buffer and stained with Coomassie Brilliant Blue [13]. The bands showing enzyme activity were located, cut and subjected to mass spectrometry analysis.

2.5. Mass spectrometry and bioinformatics analysis

Mass spectrometry was performed by tandem LC/MS/MS as described by Ref. [14]. The resulting data were analyzed by MASCOT v. 2.4 using a protein database derived from the *P. purpurogenum* genome (unpublished). Peptides showing a score >18 were considered. The amino acid sequence of each protein found was analyzed by CDD SEARCH (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) in order to establish which proteins contained domains present in esterase families. In addition, each protein identified as potential esterase was subjected to BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to search for homologies with other possible esterases. Alignment with homologous sequences was performed using CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Gene and cDNA sequences were identified using the program Augustus (http://bioinf.uni-greifswald.de/augustus/). Molecular weights and isoelectric points were estimated with EXPASY (https://www.expasy.org/); the presence of a signal peptide was determined with SignalP (http://www.cbs.dtu.dk/services/SignalP/). dbCAN (http://csbl.bmb.uga.edu/dbCAN/) was used to assign the protein sequences found to the CAZY database families (http://www.cazy.org/).

2.6. DNA preparation

P. purpurogenum was grown on 100 mL of 1% glucose for 5 days. The mycelium obtained was separated by centrifugation, frozen in liquid nitrogen and pulverized with a mortar and pestle. DNA was extracted by means of the Genomic DNA Purification kit (Fermentas). The manufacturer's instructions were followed in all procedures using kits.

2.7. PCR and overlap extension PCR

PCR was performed with Taq polymerase (Thermo Scientific) for routine assays and High Fidelity PCR Enzyme mix (Thermo Scientific) for cloning and sequencing. The conditions were as follows: initial denaturation at 95 °C for 5 min and 30 cycles of denaturation at 95 °C for 0.5 min, annealing at 59 °C for 0.5 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR