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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).

Keywords

Penicillium purpurogenum; Esterases; Pectin; Heterologous expression; *Pichia pastoris*

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.

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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 (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 ligno-cellulolytic 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 DH5 α 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 μ g 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.unigreifswald.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 products were gel purified.

Introns were eliminated using “overlap extension PCR”. In this technique, each exon is amplified with primers containing overlapping sequences between adjacent exons. Finally, all amplicons (exons) are mixed and used as template for a unique PCR reaction amplified with primers covering the 5' and 3' ends of the whole cDNA. This technique was executed as described by Topakas et al. [15]. The PCR conditions used were the same as those indicated above. Supplementary Table 1 lists the primers used for amplification of whole cDNAs and Supplementary Table 2 those used for overlap extension PCR.

2.8. Cloning and expression in *Pichia pastoris*

Cloning of the PCR products was performed in pGEM-T-Easy (Promega). Competent *E. coli* DH5α cells were transformed with the resulting plasmids. Transformed cells were plated in LB agar supplemented with 1 µl 50 mg/mL Ampicillin and 1 µl 20 mg/mL X-Gal. Plasmids from white colonies were extracted using the Plasmid DNA Mini Kit II (Omega Bio Tek) and used as templates for PCR with primers including restriction enzymes recognition sites (Supplementary Table 3) for directional cloning. The PCR products obtained were cloned in

pPICZB and the resulting plasmids were transformed into competent *E. coli* TOP10F'. Transformed cells were grown in LB plates containing 100 µg/mL Zeocin. The presence of inserts in the resulting clones was checked by PCR, and they were sequenced in both strands using the Sanger method (Macrogen Inc., Seoul, Korea).

The plasmids were linearized by means of *SacI* and transformed into *Pichia pastoris* by electroporation. Transformed clones were selected in YPD (Yeast extract Peptone Dextrose medium) agar plates supplemented with 100 µg/mL Zeocin. DNA was extracted from the clones to confirm by PCR the presence of the insert. The positive clones were grown in 20 mL BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 0.02% biotin and 1% glycerol) for 2–3 days (200 RPM, 28 °C) to obtain sufficient biomass. The cultures were centrifuged and resuspended in 20 mL of BMMY medium (glycerol is replaced by methanol) to induce enzyme production, incubating for up to 5 days. Aliquots were removed to assay for enzyme activity. Larger scale enzyme production was performed (500 mL volume) with the clones of interest.

2.9. Enzyme assays

Activity was assayed for the following substrates: MUA, p-nitrophenyl (pNP) derivatives, fluorescein diacetate, indoxyl acetate, Ruthenium Red and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). Liberation of acetic acid from pectin, pectin methyl esterase activity, cholinesterase and protease activity were also measured. Details for these assays are given in the Supplementary section (Enzyme activity assays).

2.10. Inhibition by phenylmethylsulfonyl fluoride (PMSF)

A 100 mM solution of PMSF was prepared in absolute ethanol. Enzyme activity was measured using either pNP-acetate as substrate or the pectin methyl esterase assay, with a final PMSF concentration of 2 mM.

2.11. Pectin extraction from sugar beet pulp

The procedure described by Ralet et al. [16] was used. The extraction was performed with water in order to preserve possible methyl and acetyl esters present in the pectin.

2.12. Enzyme purification using a Ni-NTA agarose column

A 4 mL nickel chelating fast flow column (GE Healthcare) was used. The column was equilibrated with a pH 8 buffer composed of 500 mM NaCl, 20 mM Tris base and 0.5 mM imidazole. The sample, in the same buffer, was loaded and the column was washed with 80 mL of the buffer. Elution was performed with increasing concentrations of imidazole (up to 500 mM) in the buffer.

2.13. Other procedures used for protein analysis

EndoH treatment (to eliminate N-glycosylations) was performed using the kit supplied by Promega. For protein quantitation, the "DC Protein Assay" by Bio-Rad was utilized. Enzyme purity was followed by SDS-PAGE [12].

2.14. Structure modelling

The amino acid sequences of the identified proteins were used to construct homology models using Schrodinger's Prime suite program (<http://www.schrodinger.com/Prime/>) after elimination of the signal peptides (detected by SignalP). The templates used are indicated for each enzyme in the Results section.

2.15. Nucleotide sequence accession numbers

The DNA sequences of the genes coding for the 3 enzymes studied have been entered in GenBank under the following numbers: RAE1: KP313786; EAN: KP313780; FAEA: KP313781.

3. Results

3.1. Identification of possible esterases in sugar beet pulp culture supernatant

In order to facilitate the identification of possible esterases, culture supernatants were subjected to partial purification by chromatography in Cibacron-Blue-Sepharose. Activity was found (using the qualitative MUA assay) in both the washing and the eluate. The concentrated pool of the active fractions obtained in the washing was subjected to chromatography in DEAE Sephadex A-25. Activity was found in the eluate fractions of this chromatogram.

Samples of the concentrated crude supernatant and of the active fractions obtained in the chromatographies were analyzed for MUA activity using zymograms. The bands showing activity were cut and subjected to mass spectrometry analysis. Fig. 1 shows, as example, the results obtained with the pool of active fractions present in the washing of the Cibacron Blue column. A total of 11 slices were analyzed by mass spectrometry.

The result of the analyses allowed the identification of 17 potential esterases, which are listed in Supplementary Table 4. Out of those 17, a group of 10 was selected for further work. This selection was based on: 1) Presence of signal peptide, 2) tentative function assigned to homologous sequences detected by BLASTp and 3) possible relation to sugar beet pulp pectin degradation (Table 1). All sequences (except *rae2*) contain introns, which were eliminated by Overlap Extension PCR. The resulting cDNA's were sequenced and compared to the sequences annotated in the genome; *lip* and *rae2* were not considered further because they showed potential stop codons in the sequence and would thus generate truncated proteins.

3.2. Heterologous expression in *Pichia pastoris* and enzyme activities shown by the clones

The sequences of the remaining 8 cDNAs coincide with the original annotations. They were linked to pPICZB, the resulting plasmids were linearized and were transformed into *Pichia pastoris*. Two clones of each protein were selected and subjected to enzyme induction by methanol. Activity of the supernatants was assayed with MUA, and clones of four of the enzymes (FAEA, FAET, RAE1 and EAN) showed activity (Supplementary Fig. 1A). No activity was shown by the negative control (*P. pastoris* transformed with the empty vector).

Next, activity of the clones' supernatants against indoxyl acetate was assayed. Cholinesterases and lipases are active on this substrate [17]. Results are shown in Supplementary Fig. 1B. Activity was detected only for RAE1 and EAN clones. In order to establish if some of the transformants produced pectin methyl esterase, the supernatants were assayed using methyl esterified pectin with different degrees of methylation. The de-esterified product (poly-galacturonic acid) reacts with Ruthenium Red giving a red spot. As shown in Supplementary Fig. 1C, only clones of PE showed activity. Since SB21, F16 and PE104 showed no activity in any of the assays attempted, they were not considered for further work. The remaining enzymes were also assayed for activity against fluorescein diacetate, a substrate for proteases, lipases, and esterases [18]. As shown in Supplementary Fig. 1D, RAE1 and FAEA are active on this substrate. Further work was performed with FAEA, RAE1 and EAN, which were purified and characterized.

3.3. Properties of RAE1

The enzyme was purified using the Ni-NTA agarose column. Fig. 2 shows the result of the purification. The calculated molecular mass is 27 kDa (including the polyhistidine tail), but a value of 32 kDa was estimated from the SDS-PAGE. Treatment with EndoH (Fig. 2) had no effect, suggesting that the enzyme may be substituted by N-glycans not susceptible to hydrolysis by EndoH or by O-glycosylation. The enzyme is active on MUA, indoxyl acetate, fluorescein diacetate and pNP-acetate. With the last substrate it shows a $K_M = 6.8$ mM, optimum pH of 7.0 and optimal temperature of 48 °C. According to the CDD database, it belongs to the SGNH hydrolase superfamily, which includes rhamnogalacturonan esterases. This activity was confirmed by assaying the enzyme with pectin extracted from sugar beet pulp, as shown in Fig. 3. The enzyme is totally inhibited after 15 min incubation with 2 mM PNSF, a typical inhibitor of serine proteases and esterases (data not shown). However, the enzyme shows no protease activity when assayed with azo-casein (data not shown).

3.4. Properties of EAN

EAN was purified by means of anion exchange chromatography (DEAE A-50). An SDS-PAGE of the purified enzyme is shown in Fig. 4. The estimated molecular mass deduced from the gel is 55 kDa, similar to the calculated value of 58 kDa. EAN is active on MUA, indoxyl acetate and pNP-acetate. Using the last substrate, a K_M of 0.78 mM, a pH optimum of 6.0 and an optimal temperature of 30 °C were estimated. According to the CDD database, EAN belongs to the esterase and lipase superfamily, which includes cholinesterases. EAN, however, shows no cholinesterase activity (when assayed with the Ellman's reagent), no activity against pNP-ferulate and does not liberate acetate from sugar beet pulp pectin (data not shown). It is active against pNP esters of fatty acids of different lengths, although with less activity than towards pNP-acetate (Fig. 5). The enzyme was inactive on acetylated birchwood xylan, even in combination with an endoxylanase and exhibited some activity on xylose tetraacetate (Fig. 6). EAN was inactive when assayed with azocasein (data not presented), thus showing that it is not a protease.

3.5. Properties of FAEA

FAEA was expressed heterologously with a polyhistidine tail, and was purified utilizing the Ni-NTA agarose column. Fig. 7 shows an SDS-PAGE of the purified enzyme, displaying a

molecular mass of about 35 kDa. When the enzyme is treated with EndoH the molecular mass goes down to about 32 kDa, near the calculated value of 31 kDa, indicating that the enzyme is N-glycosylated. The enzyme is active against MUA, indoxyl acetate, fluorescein diacetate and pNP-acetate. The K_M for this last substrate is 8.1 mM, the pH optimum is 7.0 and the temperature optimum 48 °C. The enzyme is also active against a number of pNP derivatives of fatty acids and in particular to pNP-ferulate, and shows a K_M of 0.17 mM for this substrate (Fig. 8).

4. Discussion

In the present work, 17 gene sequences of potential esterases produced by *P. purpurogenum* when grown on sugar beet pulp were identified. This has been achieved by the combination of mass spectrometry analysis of culture supernatant proteins and a search of sequences in the *P. purpurogenum* genome. The cDNA of 8 of those sequences were expressed in *P. pastoris* and three of the heterologous proteins secreted by the transformants have been purified and characterized: RAE1, EAN and FAEA.

RAE1

By means of BLASTP, four previously characterized rhamnogalacturonan esterases were identified, with sequence identities to RAE1 ranging from 71 to 28%. An alignment of the five sequences shows that the characteristic amino acids present in the SGNH hydrolases are found: four conserved blocks of residues, each with one completely conserved residue: serine, glycine, asparagine and histidine, respectively (Supplementary Fig. 2) [19]. Two of the enzymes are from fungal origin (*Aspergillus aculeatus* and *Emmericella nidulans*) and 2 from bacteria (*Bacillus subtilis* and *Bacillus halodurans*). The enzyme from *B. halodurans* is much longer; it possess a potential carbohydrate binding module in the N terminal region [20]. RAE1 is assigned by dbCAN to CAZy family CE 12, as is the case of the other homologous enzymes. A comparison of their properties is found in Table 2. Sequence identity is higher with the fungal enzymes; the bacterial enzymes have a more alkaline pH optimum, and RAE1 shows the highest optimal temperature.

Bonnin et al. [24] purified a novel pectin acetyl esterase from *Aspergillus aculeatus* and compared its properties to those of the enzyme previously purified by Ref. [21]. Both enzymes were active towards various acetylated pectins (homogalacturonan and rhamnogalacturonan fractions); therefore, the authors propose that these enzymes should be called pectin acetyl esterases. Since in sugar beet pulp pectin around 90% of the galacturonic acid and 75% of the acetyl groups belong to homogalacturonan [24], RAE1, active on sugar beet pectin, should also be considered a pectin acetyl esterase.

A model of the enzyme structure was performed using the enzyme from *Aspergillus aculeatus* (71% identity) as template (PDB 1 DEO) [19]. Supplementary Fig. 3 shows the structure obtained, typical to that of the SGNH hydrolases.

EAN

A search with BLASTP showed only one characterized enzyme with significant identity (54%) to EAN, EstA from *Aspergillus niger* [25]. This enzyme belongs to the α/β hydrolase

fold superfamily and possesses a ser-glu-his catalytic triad. In contrast to the heterologously expressed EAN, EstA is highly glycosylated. Supplementary Fig. 4 shows an alignment of both sequences and the location of the catalytic triad (also distinctive of cholinesterases [26]), and the “nucleophilic elbow” present in α/β hydrolases [27]. EstA was not assayed for cholinesterase activity, and it is inactive towards pNP-substrates. It is considered a true esterase, because it is active on vinyl esters and triacylglycerols of short chain fatty acids (2–4 carbons). A substrate specificity comparison between both enzymes is limited, due to the different substrates used for assays. The fact that EAN is active on pNP-fatty acids of different length in contrast to Est A, shows that both enzymes have different catalytic properties.

Supplementary Fig. 5 shows a model structure of EAN, which was modelled based on the structure of EstA (PDB 1UKC). Both enzymes share the catalytic triad, which in EAN is S217, H446 and E343. The presence of an active site serine in EAN was confirmed, since the enzyme is inactivated when incubated in the presence of PMSF (data not shown).

dbCAN classifies EAN in CAZy family CE 10. CAZy has withdrawn this family from its database because “the vast majority (if not all) of the members of this family are esterases acting on non-carbohydrate substrates”. EAN may, therefore, be considered tentatively either as an acetyl esterase or a true esterase.

FAEA

According to the CDD database, FAEA belongs, in the lipase-esterase superfamily, to the lipase_3 group. A BLASTP analysis shows several homologous enzymes as possible feruloyl esterases. Two feruloyl esterases characterized and with 75% identity to FAEA are found: FaeA from *Aspergillus awamori* (UNIPROT Q9P979) [28] and FaeA from *Aspergillus niger* (UNIPROT O42807) [29]. Table 3 shows some of the properties of these enzymes; FaeA from *A. niger* shows a significantly higher optimal temperature. A search of a CAZy family by dbCAN gave no results; in fact, the two FaeA from *Aspergilli* are not annotated in CAZy, although other feruloyl esterases with much lower sequence identity to the enzymes above are classified as CE 1 (i.e. FAEA shows only 21% identity with a CE 1 feruloyl esterase (FaeC, UNIPROT Q5B2G3) from *Aspergillus nidulans*). The feruloyl esterases discussed here deserve, therefore, to be assigned to a new CE family by CAZY.

Supplementary Fig. 6 presents an alignment of FAEA with the feruloyl esterases mentioned and with several fungal lipases (*Thermomyces lanuginosus*, UNIPROT O59952, 36% identity; *Rhizomucor miehei*, UNIPROT P19515, 35% identity; *Rhizopus oryzae*, UNIPROT P61872, 30% identity; *Rhizopus niveus*, UNIPROT P61871, 29% identity). The alignment shows that all sequences conserve the catalytic triad (Ser-His-Asp) and the nucleophilic elbow (GX SXG) as expected from enzymes belonging to the lipase_3 group. A proposed structure of FAEA was obtained using *Aspergillus niger* FaeA (PDB 1UWC, [30]) as model (Supplementary Fig. 7). The model shows the location of the catalytic triad (ser 215, his 268 and asp 154) and the nucleophilic elbow. The presence of an active site serine was experimentally confirmed: the enzyme is totally inhibited by 2 mM PMSF (data not shown). A lack of activity on azocasein indicates that the enzyme is not a protease (data not shown). In short, FAEA can be identified as a feruloyl esterase.

In conclusion, three esterases present in the culture supernatant of *P. purpurogenum* grown on sugar beet pulp have been identified, expressed heterologously in *P. pastoris*, purified and characterized. They likely participate in the biodegradation of the pectic component of sugar beet pulp in synergy with other hydrolases and lyases. FAEA (feruloyl esterase) may participate in the liberation of ferulic and other cinnamic acid residues present in RG1. RAE1, a pectin acetyl esterase will hydrolyze acetate residues esterifying galacturonic acids both in homogalacturonan and RG1. Less clear is the function of EAN; it may act as an acetyl esterase on short chain fragments of homogalacturonan or RG1 previously degraded by other hydrolases or lyases. These enzymes may be valuable tools for a detailed study of pectin degradation.

Results of the characterization of the heterologously expressed FAET (feruloyl esterase) and PE (pectin methyl esterase) from crude extract supernatants have been published elsewhere [31].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.carres.2017.03.014>.

Fig. 1. Zymogram of the pool of active fractions obtained in the washing of the Cibacron-Blue Sepharose column. Lanes: 1, Coomassie staining; 2, zymogram showing activity on MUA; 3, molecular weight standards. The symbols on the left indicate the gel slices that were cut and subjected to mass spectrometry.

Fig. 2. SDS-PAGE of purified RAE1. Lane 1, molecular weight standards; lane 2, purified enzyme; lane 3, purified enzyme treated with EndoH.

Fig. 3.

Liberation of acetate from sugar beet pectin by RAE1. 1% sugar beet pectin was incubated with 1.5 μg RAE1 in phosphate buffer pH 5.8 for 16 h at 28 °C. As control, alkaline hydrolysis was performed with 2 M NaOH. Experiments performed in triplicate. Bars show standard deviation.

Fig. 4. SDS-PAGE of the purified EAN. Lane 1: purified enzyme; lane 2, molecular weight standards.

Fig. 5.

Activity of EAN against a set of pNP-fatty acid derivatives of different lengths. Incubation: 90 min at 28 °C; substrate concentration: 10 mM. 1: pNP-acetate; 2: pNP-butyrate; 3: pNP-caprylate; 4: pNP-dodecanoate; 5: pNP-myristate; 6: pNP-palmitate. Experiments performed in triplicate. Bars show standard deviation.

Fig. 6.

Activity of EAN on acetylated xylan and xylose tetraacetate. The enzymatic liberation of acetate was measured after incubation for 24 h at 28 °C. Alkaline hydrolysis was performed with 2 M NaOH for 24 h at 60 °C. 1: activity of EAN on acetylated xylan; 2: alkaline hydrolysis of acetylated xylan; 3: activity of EAN on xylose tetraacetate; 4: alkaline hydrolysis of xylose tetraacetate. Experiments performed in triplicate. Bars show standard deviation.

Fig. 7. SDS-PAGE of the purified FAEA. Lanes 1 and 4, molecular weight standards; lane 2, purified FAEA; lane 3, purified FAEA treated with EndoH.

Fig. 8.

Activity of FAEA against a set of pNP-derivatives. 2.5 mM of each substrate was incubated with 0.05 μ g FAEA for 4 h at 28 °C. 1: pNP-acetate; 2: pNP-butyrate; 3: pNP-caprylate; 4: pNP-decanoate; 5: pNP-dodecanoate; 6: pNP-myristate; 7: pNP-palmitate; 8: pNP-ferulate. Experiments performed in triplicate. Bars show standard deviation.

Table 1

Esterases selected for heterologous expression in *P. pastoris*.

Identification	Name	BLASTP first hit (% identity)
0.149–81	FAEA	<i>Penicillium oxalicum</i> hypothetical protein (80%)
1.43–45	FAET	<i>Penicillium oxalicum</i> hypothetical protein (76%)
0.146–90	LIP	<i>Penicillium oxalicum</i> hypothetical protein (74%)
0.164–103	RAE1	<i>Penicillium chrysogenum</i> hypothetical protein (79%)
2.36–17	RAE2	<i>Penicillium chrysogenum</i> hypothetical protein (69%)
2.113–11	EAN	<i>Oidiodendron maius</i> hypothetical carboxyl esterase (66%)
0.107–5	SB21	<i>Penicillium oxalicum</i> hypothetical protein (81%)
2.61–14	PE	<i>Aspergillus kawachi</i> hypothetical pectin esterase (77%)
0.104–113	PE104	<i>Penicillium oxalicum</i> hypothetical pectin methyl esterase (83%)
5.194–4	F16	<i>Penicillium oxalicum</i> hypothetical carbohydrate acetyl esterase (62%)

Identification: Sequence and scaffold numbers annotated in the *Penicillium purpurogenum* draft genome.

Table 2

Comparison of the properties of RAE1 with those of other characterized rhamnogalacturonan esterases.

Organism (UNIPROT code)	References	% identity with RAE1	pH optimum	Optimal temperature (°C)	K _M (mM)
<i>Penicillium purpurogenum</i>	This work	100	7 ^a	48 ^a	6.8 ^a
<i>Aspergillus aculeatus</i> (Q00017)	[21]	71	6 ^b	40 ^a	ND
<i>Emertella nidulans</i> (QBAA2)	[22]	69	ND	ND	ND
<i>Bacillus subtilis</i> (O31523)	[23]	30	8.5 ^a	35 ^a	2.8 ^a
<i>Bacillus halodurans</i> (Q9KDU6)	[20]	28	8 ^a	40 ^a	14.1 ^a

ND: not determined.

^a pNP acetate used as substrate.

^b Modified hairy regions from apple pectin used as substrate.

Table 3

Comparison of the properties of FAEA with those of two other fungal feruloyl esterases.

Enzyme origin (UNIPROT code)	References	% identity with FAEA	pH optimum	Optimal temperature (°C)
FAEA	This work	100%	7 ^a	48 ^a
<i>Aspergillus awamori</i> (Q9P979)	[28]	75%	5 ^b	45 ^b
<i>Aspergillus niger</i> (O42807)	[29]	76%	5 ^c	55–60 ^c

^aSubstrate: pNP-acetate.

^bSubstrate: 5-O-(*trans* feruloyl) arabinofuranose.

^cSubstrate: methyl ferulate.