Trichoderma harzianum Lip1 gene

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Introduction and aims

An extraordinary panoply of cell-wall degrading enzymes has been related to the mycoparasitism process of *Trichoderma* sp. However, the role of lipolytic enzymes in this process is less known. The aim of this study is to characterize the first extracellular triacylglicerol lipase described in *T. harzianum* .

The nucleotide sequence of *Lip1* gene from *T. harzianum* CECT 2413 (T34) can be accessed in EMBL database (AM180877.1), including the 5' upstream and the 3' downstream regions.

Lip1 open reading frame (ORF) has 1667 bp, encoding a predicted protein of 532 amino acids (Lip1), that can be accessed in UniProtKB (B0B099_TRIHA).

Material and Methods

T. harzianum Lip1 gene was characterized by bioinformatic analysis such as the corresponding deduced protein Lip1.

Southern Blot experiments were made to determine *Lip1* copy number in genomic DNA of *T. harzianum*, as well as the presence of homologous genes in the genomes of *T. atroviride, T. reesei and T. virens.* To assess the presence of homologous genes in the genomes *of T. atroviride, T. reesei* and *T. virens*, the genomic DNAs of these species were digested with the endonucleases *Sac* II, *Xho* I, *Not* I and *Xba* I. In the copy number detection assay, the restriction endonucleases *Cla* I, *Sac* I, *Stu* I, *Xba* I, *Xho* I, *Sal* I, *Sac* II, *Not* I + *Nco* I and *Stu* I + *Pvu* II were used. Some of them cut once or twice in the entire gene sequence, including the known promoter and terminator regions: *Cla* I, *Not* I, *Sac* I, *Sal* I and *Stu* I, *Nco* I, *Sac* II, PvuII), others did not cut it (XbaI and XhoI).

Hybridization occurred at 62 ºC and 0.5XSSC buffer; 0.1% SDS (in other *Trichoderma* species) or at 68 °C in 0.1XSSC buffer; 0.1% SDS, in *T. harzianum* using as probe a 674bp fragment of the Lip1 gene tagged with digoxigenin.

Lip1 was cloned in *Pichia pastoris* GS115 by electroporation using the constitutive expression vector pIB2 (Sears et al., 1998), and lipolytic activities of selected transformants were evaluated.

Plate screening assays with tributyrin and Rhodamine were used for lipolytic activity detection, but this activity also was determined spectrophotometrically by measuring the release of *p*-nitrophenol, using *p*-NP butyrate (C_4) and *p*-NP palmitate (C_{16}) as substrates. In plate screening assays two different base culture media were used, one similar to YEPD, but with addition of 1% tributyrin, and varying the percentage of glucose (0; 0.3; 0.5; 1 and 2%), denominate Tributyrin Agar (TA); the second one designed Lipase Detection Agar (LDA), was composed by a base culture medium with 0.5% peptone, 0.3% yeast extract, 1.5% agar, pH adjusted to 6.0. After autoclaving, 10 ml of 10X YNB and 0.2 ml of 500XB were added per 100 ml. Glucose added varied from 0 to 10 ml of 10XD glucose, according to the modalities tested (0; 0.3; 0.5; 1 and 2% glucose). To nine parts of this base media were added one part of a lipidic emulsion composed by an oil at 30% (olive oil, corn oil or soybean oil), 12% arabic gum and rhodamine (at 0.02% p/v in LDA). All assays were done in triplicate with three replicates.

Results and Discussion

In Figure 1 are represented the results of the Southern Blot analysis in *T. harzianum:* a fragment of 3645 bp corresponds to the *Nco* I + *Not* I combination in the gDNA of *T. harzianum*, and to that of *Stu* I + *Pvu* II, a fragment of 3306 bp. In turn, *Sac* II originates a fragment of 3229 bp. In all reactions only single fragments have been detected, and it can be deduced that *Lip1* is a unique copy gene in the genome of *T. harzianum*. In the assay with other species of the genus, as represented in Figure 2, the probe only hybridize with *T. harzianum,* indicating that there are no genes homologous to *Lip1* in these species of the genus *Trichoderma*, of which the genome sequence is known.

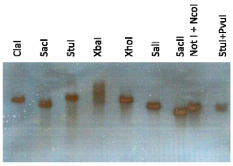


Figure 1- Southern Blot analysis of the *Lip1* gene in *T. harzianum*. Restriction endonucleases used: ClaI, SacI, StuI, XbaI, XhoI, SalI, SacII (3229pb), NotI + NcoI(3645bp) and StuI + PvuII (3306pb). Hybridization occurred at 68 ° C in 0.1XSSC buffer; 0.1% SDS, using as probe a 674bp fragment of the Lip1 gene.

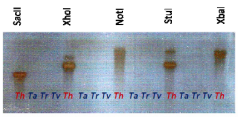
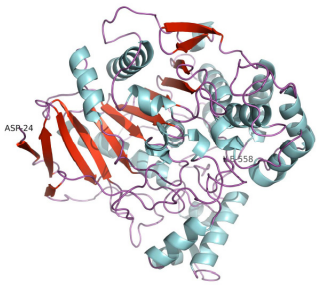


Figure 2- Southern Blot of the *Lip1* gene in different species of the genus *Trichoderma*. The genomic DNA sequence in the gel was: *T. harzianum* (Th), *T. atroviride* (Ta), *T. reesei* (Tr) and *T. virens* (Tv). Restriction endonucleases SacII, XhoI, NotI, StuI and XbaI were used. Hybridization occurred at 62°C in 0.5XSSC buffer; 0.1% SDS, using a 674 bp fragment of the *Lip1* gene as a probe. Hybridization with the probe only occurred in *T. harzianum*.

Lip1 has a carboxylesterase type-B signature, with a serine active site (PROSITE PS00122) (Sigrist et al., 2002). As in lipases and serine proteases, the catalytic triad of esterases is formed by three amino acids: a serine, a glutamic or aspartic acid, and a histidine. Sequence around the serine-containing active center serine is well preserved, and is used as a signature pattern: F-[GR]-G-x(4)-[LIVM]-x-[LIV]-x-G-x-S-[STAG]-G. As secondary pattern was selected a conserved region located at the N-terminal region, which contains a cysteine involved in a disulfide bond, the sequence is [EDA]-[DG]-C-L-[YTF]-[LIVT]-DNS]-[LIV]-[LIVFYW]-x-[PQR]. In Lip1 are present the sequences FGGDPDKVTLWGFSAG, and EDCLTLNVQRP, in the amino acid positions 216-231 and 115-125. The serine at the active center of Lip1 corresponds to residue 229, with a relative position similar to that existing in other lipases and a primary structure coincident with the consensus G-x-S-x-G, described as an active center of lipases. The other components of the catalytic triad are the residues E361 and H474. The oxyanion hole, critical for catalysis, is located in residues 134-144.

In Figure 3A are represented the three-dimensional structural prediction made on the Phyre2 server (Kelley & Sternberg, 2009), based on the homology of Lip1 with the crystallized protein 4BE4 of the fungus *Ophiostoma piceae* in closed conformation (with access code Q2TFW1 in the "Protein Data Bank") (Gutiérrez-Fernández et al., 2014). This prediction reveal that Lip1 "lid" region is constituted by an α-helix (residues 99-107) flanked by two "loops" that end in a disulfide bridge (Cys 83-Cys117). Figure 3B shows a detail of Lip1 evidencing the catalytic triad and the oxyanion hole.



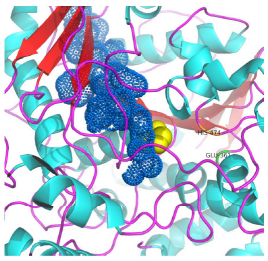


Figure 3A - Graphical representation of the prediction of the 3-D structure of Lip1. The structural prediction has been made on the Phyre237 server based on the homology of Lip1 with the crystallized protein 4be4 of *Ophiostoma piceae* in closed conformation. Red wavy ribbons with arrowhead represent the regions with β-sheet conformation and the blue spiral ribbons those with α-helix conformation. The areas of loops or turns are represented as tubular purple elements. The asparagine residue (Asp24) indicates the amino-terminal end, the isoleucine (Ile558) the carboxyl-terminal. The representation has been made from the data corresponding to the theoretical elucidation using the PyMol program.

Figure 3B - Detail of Lip1 evidencing the catalytic triad and the oxyanion hole.The active center residues are: serine (S229), represented in yellow; glutamic acid (E361), represented in green, and histidine (H474), represented in orange. The oxyanion hole is formed by the residues represented by blue points.

The results of the screening plate assays with the most promising clones obtained after transformation of *P. pastoris* are represented in Table 1, and in Figures 4 and 5. Amongst them, pLJ4 seemed to have more lipolytic activity in the same experimental conditions. In this clone are observed halos due to esterase activity in TA media, showing its esterase activity. The ADL medium with soybean oil gave the highest fluorescence intensity that is characteristic of lipase fatty acid utilization in all clones tested, but more intense and earlier in pLJ4. Figure 6 shows the cinetic of the hydrolisis reaction using p-nitrophenyl butyrate as the substrate using cellular extracts of the clone pLJ4. All of these results are consistent , indicating that Lip 1 has affinity for short chain fatty acids (C<10) and soluble substrates.

Table 1 - Diameter of the colonies and lipid degradation halos (cm) obtained in the Tributyrin Agar plate assays.

	GS115		pLJ1		pLJ4		pLJ17	
Day	Halo	Colony	Halo	Colony	Halo	Colony	Halo	Colony
1	_	0,4	0,6	0,4	**0,7**	0,4	0,6	0,4
2	_	0,4	0,9	0,4	**1,2**	0,4	0,9	0,4
3	_	0,5	1,5	0,5	**1,8**	0,5	1,4	0,5
5	_	0,5	>2	0,5	**>2**	0,5	1,8	0,5

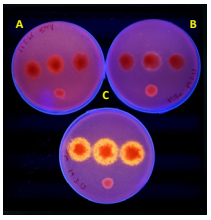


Figure 4 - Fluorescence halos due to lipase activity of clones pLJ17, pLJ4 and pLJ1, 20 days after inoculation in LDA medium with glucose at 0.3%, and 3% olive (A), corn (B) and soybean oils (C), respectively.
In the image, by plate, from left to right: pLJ17, pLJ4, and pLJ1, respectively; At the bottom, the GS115 yeast, used as control. Each colony was originated from 4µl of a suspension with ~ $5x10^7$ cells / ml.

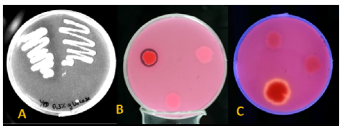


Figure 5 - Halos of esterase activity (A and B) and fluorescence due to lipase activity of clone pLJ4. **A - Tributyrin Agar** (0.3% glucose, in the left side growth of pLJ4, in the right side growth from *P. pastoris* GS115); **B – Rhodamine Tributyrin Agar** modified by the addition of rhodamine B at 0.02%; **C - LDA,** with 3% olive oil as a lipid substrate. In all modalities the growth observed on each plate that do not present haloes or fluorescence correspond to the untransformed yeast (GS115) or to the transformed with the pIB2 plasmid but without insert, used as negative controls. In the two plates represented in B and C the growth originated from 3µl of a suspension with ~ $5x10^7$ cells / ml.

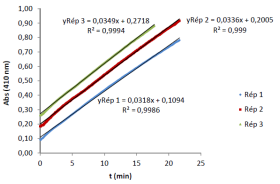


Figure 6 - Absorbance values obtained with cellular extracts of clone pLJ4 using p-nitrophenyl butyrate as the substrate.

References

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