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Artículo original de investigación

Cloning and expression of a hypothetical Loosenin from *Neurospora crassa*

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Abstract

Loosenin is a protein recently described which presents amorphogenic activity on cellulose. It was isolated from the Basidiomycete *Bjerkandera adusta* and it enhances sugar release from cellulosic fibers treated previously with it and then subjected to cellulase treatment. It can also bind to other polysaccharides like chitin and xylans but presents no hydrolytic activity itself. Blast analysis using the loosenin amino acid sequence retrieved a sequence from the *Neurospora crassa* genome that showed 59% similarity. In this work, we cloned, expressed and partially characterized a putative loosenin from *N. crassa*, since these proteins have a potential for pretreatment of lignocellulosic materials.

Keywords: *Amorphogenesis, bioethanol, lignocellulose, loosenin*.

Resumen.

La loosenina es una proteína descrita recientemente que presenta actividad amorfogénica sobre la celulosa. Se aisló a partir del hongo Basidiomiceto *Bjerkandera adusta* y facilita el desprendimiento de azúcares reductores a partir de fibras celulósicas tratadas previamente con ella y posteriormente expuestas a tratamiento con celulasas. Puede unirse a otros polisacáridos como quitina y xylanos aunque no presenta actividad hidrolítica por sí misma. En un análisis Blast empleando la secuencia de aminoácidos de la loosenina se localizó una secuencia del genoma de *Neurospora crassa* que mostró 59% de similitud. En el presente trabajo, clonamos, expresamos y caracterizamos parcialmente una posible looenina de *N. crassa*, debido a que estas proteínas tienen potencial para el pretratamiento de materiales lignocelulósicos.

Palabras clave: *amorfogénesis, bioetanol lignocelulosa, loosenina*

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Introduction

Lignocellulose is the most abundant biomass source; its degradation to simple sugars has been considered a viable way to produce bioethanol (Gray *et al.* 2006). However to use cellulose, molecular disorder of crystalline cellulose must be increased in the fiber network of lignocellulose, and expose the glycosidic chains that are inside of the microfibril, this process is called amorphogenesis (Arantes and Saddler 2010). Expansins are plant proteins necessary in the process of plant cell wall expansion but do not have hydrolytic activity by themselves. It is proposed that its mechanism disrupts microfibrils by breaking hydrogen bonds, destabilizing the crystalline structure of cellulose or the interactions between cellulose and hemicellulose (Sampedro and Cosgrove 2005). These proteins have two domains, preceded by a signal peptide. DPBB (double psi beta barrel) domain or Domain I, has a homology with a GH45 family; and Domain II which has an immunoglobuline-like fold and its function is still unknown (Sampedro and Cosgrove 2005).

There are four expansin families that are well described: α-expansins, β-expansins, α expansin-like and β expansin-like proteins: EXPA, EXPB, EXLA, EXLB respectively. Between the members of these families, only 20 to 40% of identity is shared at the level of amino acid sequence, and the highest conservation is found in the DPBB domain (Sampedro and Cosgrove 2005). Another group of expansins was described of organisms that are not plants; this group was called EXLX. Examples of these proteins are EXLX1 from *Bacillus subtilis* (Kim *et al.* 2009), or expansin like genes in *Dictyostelium discoideum*, (Darley *et al.* 2003), and others. Saloheimo *et al*., described a protein with an expansin-like activity in the fungus *Trichoderma reesei* (Saloheimo *et al.,* 2002). This protein (SWO1) has one domain with homology to Domain II of plant expansins and when expressed in *Saccharomyces cerevisiae* it showed swollen regions in cotton fibers treated with SWO1 but without liberation of reducing sugars.

In our group we previously described a novel protein with expansin-like activity from the Basidiomycete *Bjerkandera adusta*, it was called loosenin (LOOS1). This protein binds to polysaccharides and has only one domain with a DPBB fold. It was demonstrated that previous treatment of lignocellulosic materials (cotton fibers and Agave bagasse) with loosenin, enhances sugar release from these fibers after treatment with cellulases (Quiroz-Castañeda *et al.* 2011).

Nowadays, the biological role that loosenin or other expansin-like proteins could play in fungi is not known; one possibility is that they could be involved in its own cell wall remodeling to allow growth of the hyphae, a second one, that is part of lignocellulolytic metabolism of fungi. In a bioinformatic analysis, we found one sequence of *Neurospora crassa*, denominated N2, with 59% of similitude to LOOS1. Because *N. crassa* is considered a model organism, with a lot of genetic and molecular tools, we cloned and expressed this sequence (N2) in *Kluyveromyces lactis*, to evaluate its amorphogenic activity.

Methodology

To find structural templates we used PHYRE web server version 0.2, the models were made with Swiss Model (http://swissmodel.expasy.org) with every single template, and visualized with PyMOL viewer (DeLano Scientific LLC, 2006) as separate. The structural overlap with EXLX1 from *Bacillus subtilis* was chosen as the best model. The signal peptide prediction of the sequence from *Neurospora crassa* was made with the web server SignalP

(http://www.cbs.dtu.dk/services/SignalP/).
To identify the strictly conserved and conserved amino acids described by Kerff et al. (Kerff *et al.* 2008), we used the three sequences in the alignment from PHYRE, two sequences from *Neurospora crassa*, and the LOOS1 sequence.
The wild type strain of *Neurospora crassa* was kindly provided by Dr. Wilhelm Hansberg of the Institute of Physiology, UNAM. The mycelium was grown in mineral medium (7 mg/L, $CuSO_4 \cdot 5H_2O$; 18 mg/L, $FeSO_4 \cdot 7H_2O$; 500 mg/L, $MgSO_4 \cdot 7H_2O$; 10 mg/L, $ZnSO_4$; 50 mg/L, KCl; 1 g/L, K_2HPO_4) plus 2% bran flakes as a carbon source; after two days of growth the mycelium was collected. The total RNA was obtained as described by Sokolovsky et al. (Sokolovsky *et al.* 1990). The RNA was used as a template to amplify cDNA with SuperScript™ III RT/ Platinum® Taq (Invitrogen) according to the kit specifications. The sequence of interest N2 (accession number XP_959591.1) was amplified with specific oligonucleotides Fwd: 5' AAGCTTGAAAAAAATGCTCTTCCAAC CCATCCTGAC 3' and Rev 5' CTCGAGCTAAACCCAATCCCAACTCC CCT 3'), and the expected fragment of 472 pb was purified from agarose gels and cloned in pGEM-T vector (Invitrogen), and then was sucloned in the expression vector pKLAC1 (Biolabs) in the restriction sites *Hind*III and *Xho*I (underlined in the oligonucleotide sequences). The expression cassette was liberated by digestion with *Sac*II, and this DNA was used to transform the yeast, with the lithium acetate method. Positive clones were selected in YCB Centrum + Acetamide 1 mM medium, and the expression was induced in YNB + Galactose 1% medium. After two days of growth the supernatant was collected, and concentrated to 20 ml by a membrane of 10 kDa (Polyethersulfone (PES), Sartorius Stedim Biotech), this volume then was centrifuged in a cut-off membrane of 30 kDa (Vivaspin 20, PES 30 kDa MWCO, GE Healthcare), and finally again in a cut-off membrane of 10 kDa (Vivaspin 20, PES 10 kDa MWCO, GE Healthcare). All the fractions obtained were observed in a SDS-PAGE 15% denaturing gels, stained with coomassie blue 0.125% (Brilliant Blue R-250, Sigma). Amorphogenic activity was evaluated incubating the corresponding fraction of concentrated protein with cotton fibers (previously pretreated with NaOH 25%, for 15 min at 4°C, and washed three times with distilled water) in acetate buffer pH 5.0; 8 hours at room temperature, then were sonicated (Ultrasonic cleaner 1510, Branson) for 5 min, after incubation and the cotton fibers visualized by contrast phase microscopy (Zeiss).

Results

Bioinformatic analysis

The N2 amino acid sequence was used for fold recognition with PHYRE, which identified the same structural templates that were identified for LOOS1 from *Bjerkandera adusta*: EXLX1 from *Bacillus subtilis* [PDB:2BH0], the homologue pollen allergen PHL P1 N-terminal domain from *Phleum pratense* [PDB:1N10] and an EXPB and group-1 allergen from maize [PDB:2HCZ]. Tridimensional structure was determined *in silico* with Swiss Model (http://swissmodel.expasy.org), with EXLX1 as template, and the structural superposition of the model N2 was observed in PyMOL viewer (DeLano Scientific LLC, 2006) (Figure 1). Also a signal peptide of 25 amino acids was predicted with the web server SignalP (http://www.cbs.dtu.dk/services/SignalP/).

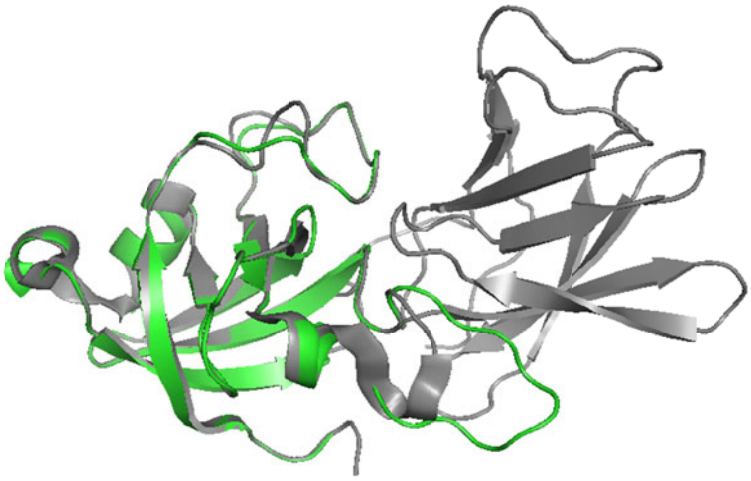

Figure 1 Structural superposition of N2 model (green) with EXLX1 (gray). The DPBB fold was observed in LOOS1 with the same template.

Alignment of sequences with polysaccharide recognizing proteins like EXLX1, 1N10, 2BH0, SWO1, and LOOS1 allowed us to identify strictly conserved amino acids, and conserved amino acids involved in binding polysaccharides (Figure 2). There are no changes in the strictly conserved amino acids T31 and D105 in LOOS1, but in the conserved amino acid T92 in LOOS1 change for V114 in N2, and D103 in LOOS1 change for G124 in N2 sequence.

```
LOOS1   -------VVG FVIA-APTGL QHGDGTFYA- -------TGL --GACGKVN- VDT----DMI AAVSHQFFDS 60
1n10    --PKVPPGPN ITAT-YGDKW LDAKSTWYGK PTGAGPKDNG --GACGYKD- VDKPPFSGMT GCGNTPIFKS 64
2bh0    ---------- -----AYDDL HEGYATYTG- -------SGY SGGAFLLDPI PSD----MEI TAINPADLNY 44
2hcz    ---KVPPG-N ITTN-YNGKW LTARATWYGQ PNGAGAPDNG --GACGIKN- VNLPPYSGMT ACGNVPIFKD 66
N1      ASPQAVESST TESTSSTSSS HNGDLTYY-- ------DVGL --GACGFDD- NGLDMSDNI- VALSHEMMGT 201
N2      ---PIAIREV STTETLDPRS FSSRITWYN- -------TGL --GACG---- -TYSNDGQLV VALNHDQFDP 83

LOOS1   FPGAGANPNN NPICGRTATV HHGS------ KTIKVQITDR CGGCKGATDL DFSPSAFNKL -------ADP 117
1n10    G---------- -RGCGSCFEI KCTKPEACSG EPVVVHITDD NEEPIAPYHF DLSGHAFGAM AKKGDEQKLR 124
2bh0    GGVK------ AALAGSYLEV EGPK------ GKTTVYVTDL YPEGARG-AL DLSPNAFRKI -------GNM 94
2hcz    G---------- -KGCGSCYEV RCKEKPECSG NPVTVYITDM NYEPIAPYHF DLSGKAFGSL -------AKP 119
N1      QSNGNPY--- --CNRKVIIK ANG------- KTVTATVRDK -CMGCAFNDI DVSKKAFLDV -----FGALT 253
N2      STPNGNPNRN -SLCGRRIRV NA------NG RSVTVTLVDR -CTQCPYGGL DLSPAAFSVL ASTSVGVVQG 145

LOOS1   SFGRIPITWT LD                                                          129
1n10    SAGELELQFR RVKCKYPEG                                                   143
2bh0    KDGKINIKWR VVKAPITGNF TYRIKEGSSR WWAAIQVRNH KYPVMKMEYE KDGKWINMEK MDYNHFVSTN 164
2hcz    GLNDKIRHCG IMDVEFRRVR CKYPAGQKIV FHIEKGCNPN YLAVLVKYVA DDGDIVLMEI QDKLSAEWKP 189
N1      DGRKKVEWAFSD                                                           265
N2      SWDWV                                                                  150
```

Figure 2 Sequence alignment of three structural templates, LOOS1, and two sequences of *Neurospora crassa*. The residues conserved, and strictly conserved are highlighted in yellow and cyan respectively. Modified from (Quiroz-Castañeda *et al.* 2011).

Experimental results

To clone the N2 gene sequence from *Neurospora crassa,* total mRNA was extracted to use as a template in a RT-PCR with specific oligonucleotides. The amplification product of 472 pb was cloned in a standard cloning vector, later was digested for subcloning in pKLAC1. The sequence of signal peptide from fungus was conserved, so the α-MF from pKLAC1 vector was removed. The yeast *Kluyveromyces lactis* was transformed with the expression cassette digested with *Sac*II (Figure 3).

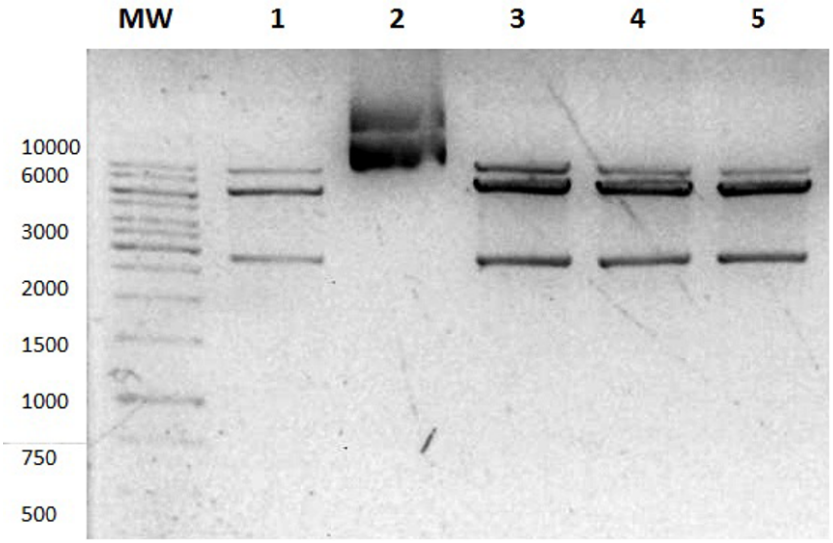

Figure 3 Construction pKLAC1::N2, digested with *Sac*II to liberate the expression cassette. Lane 1: pKLAC1/*Sac*II; lane 2: pKLAC1::N2 construction without digestion; lane 3-5: pKLAC1::N2/*Sac*II.

The positive colonies were cultivated in YNB+Galactose 1% to induce the expression. The supernatant was collected and the total protein was concentrated by ultrafiltration. Denaturing gels SDS-PAGE 15% were made and allowed us to identify a protein of the predicted size, about 16 kDa (Figure 4). However, this band was observed in the fraction corresponding to proteins larger than 30 kDa, but only in the transformant yeast supernatants. An explanation to this behavior could be that the protein is associated with other proteins of larger molecular weight, or forming oligomers among itself. This protein however is not present in the control strain supernatants.

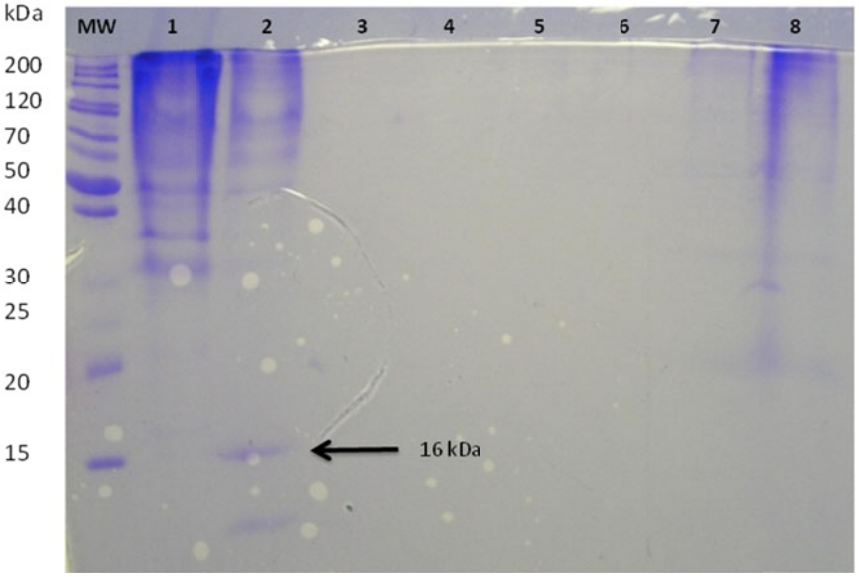

Figure 4 Concentrated protein from supernatant of *Kluyveromyces lactis* grown in YNB+Gal 1% medium. Lane 1: fraction of protein >30 kDa from *K. lactis WT*; lane 2: fraction of protein >30 kDa from *K. lactis::N2*; lane 3: fraction <30 kDa and >10 kDa *K. lactis WT*; Lane 4: fraction <30 kDa and >10 kDa; lane5: fraction <10 kDa *K. lactis WT*; lane6: fraction <10 kDa *K. lactis:: N2*; lane 7: yeast lysate *K. lactis WT*; lane 8: yeast lysate *K. lactis::N2*.

The selected fraction was evaluated on it disrupting activity on cotton fibers, where bubble-like structures were observed (Figure 5).

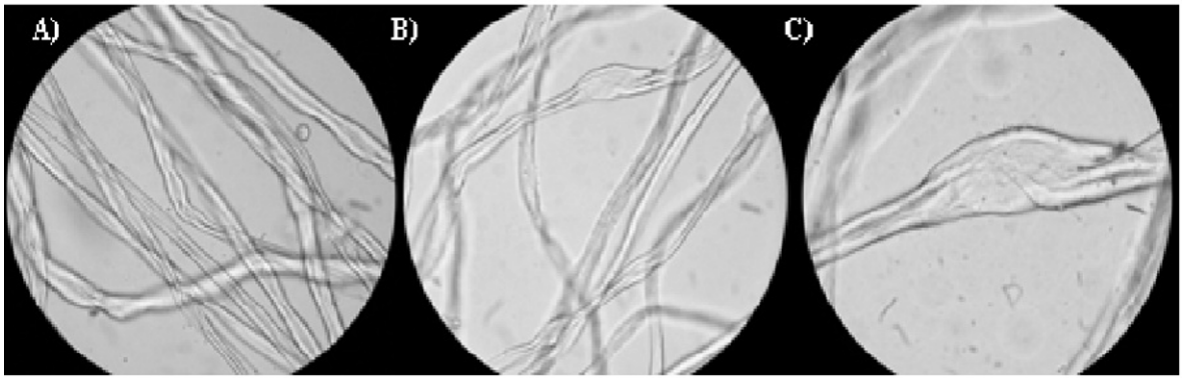

Figure 5 Representative micrographs of qualitative assay, with cotton fibers. a) Cotton fibers incubated with fraction >30 kDa of protein concentrated from *K. lactis WT* as a negative control (40X). b) Cotton fibers incubated with fraction >30 kDa of protein concentrated from *K. lactis::N2* (40X) c) Cotton fibers incubated with fraction >30 kDa of protein concentrated from *K. lactis::N2* (100X).

Conclusions

The tridimensional structure of N2, a putative ortholog of LOOS1, was predicted *in silico*, with EXLX1 as a template, and DPBB fold was confirmed. Strictly conserved and conserved amino acid residues were identified by an alignment with other sequences of proteins with expansin like activity that have been evaluated experimentally; this suggests that N2 could be a polysaccharide binding protein. The DNA sequence from *Neurospora crassa* was amplified and cloned in the expression vector pKLAC1 using the native signal peptide from the fungus. A protein about 16 kDa was observed in the SDS-PAGE denaturing gels and this fraction of enriched protein was evaluated for disrupting or amorphogenic activity and bubble-like structures were observed by contrast phase microscopy, when we used the control strain supernatants, we did not observe these structures, strongly suggesting that N2 is indeed an ortholog of *B. adusta* LOOS1.

Acknowledgements

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