Aspartyl protease from *Trichoderma harzianum* CECT 2413: cloning and characterization

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A gene that encodes an extracellular aspartyl protease from *Trichoderma harzianum* CECT 2413, *papA*, has been isolated and characterized. Based on several conserved regions of other fungal acid proteases, primers were designed to amplify a probe that was used to isolate the *papA* gene from a genomic library of *T. harzianum*. *papA* was an intronless ORF which encoded a polypeptide of 404 aa, including a prepropeptide at the N-terminal region formed by one putative signal peptide, a second peptide which could be cleaved to activate the enzyme and the active protease of calculated 36.7 kDa and pI 4.35. Northern experiments indicated that *papA* gene was pH regulated, repressed by ammonium, glucose and glycerol, and induced by organic nitrogen sources. The promoter possessed potential AreA, PacC and MYC sites for nitrogen, pH and mycoparasitism regulation respectively, but lacked potential CreA sites for carbon regulation. IEF and zymograms indicated that PAPA was a pepstatin-sensitive aspartyl protease of pI 4.5. Transformants from *T. harzianum* CECT 2413 cultivated in yeast extract-supplemented medium overexpressed *papA* and had a fourfold increase in protease activity compared to the wild-type, while transformants that overexpressed the β -1,6-glucanase gene *bgn16.2* and *papA* had an additional 30% increase in β -1,6-glucanase activity compared to *bgn16.2* single transformants. Overexpression of both genes in ammonium-supplemented medium did not result in higher levels of PAPA and/or BGN16.2 proteins. These results indicated that both PAPA and β -1,6-glucanase undergo proteolysis in ammonium-supplemented medium but PAPA is not responsible for β -1,6-glucanase degradation.

Keywords: fungal proteases, nitrogen carbon and pH regulation, protein overproduction, proteolysis

INTRODUCTION

Fungal proteases play several roles in metabolism, nutrition and morphogenesis (Archer & Peberdy, 1997). Utilization of proteins requires extracellular digestion by proteases to hydrolyse the proteins to free amino acids. Proteases are also involved in post-secretional processing of extracellular hydrolytic enzymes (Chen *et al.*, 1993; Archer & Peberdy, 1997; Bussey, 1988). Some of these proteases are very harmful, especially to heterologous proteins (Archer, 2000), but also to homologous ones (Delgado-Jarana *et al.*, 2000). Some

authors have found a correlation between high levels of extracellular acidic proteases in *Trichoderma* species and the appearance of proteolytic cellulase (Hagspiel *et al.*, 1989; Haab *et al.*, 1990), β -glucosidase (Kubicek-Pranz *et al.*, 1991), chitinase (Margolles-Clark *et al.*, 1996) or β -1,6-glucanase (Delgado-Jarana *et al.*, 2000) degradation products.

The amount of proteases can be diminished by modifying the cultivation procedure (Delgado-Jarana *et al.*, 2000) or by isolating protease-deficient strains (Berka *et al.*, 1990; Moralejo *et al.*, 2000; Archer, 2000; van den Hombergh *et al.*, 1997a, b). Deletion of an aspartic proteinase gene decreased the degradation of the secreted calf chymosin by *Aspergillus niger* (Dunn-Coleman *et al.*, 1991) and of thaumatin by *Aspergillus awamori* (Moralejo *et al.*, 2000), while deletion of a

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Table 1. *Trichoderma* strains used in this study

Strain	Species	Description	Source/reference
CECT 2413	<i>T. harzianum</i>	WT	CECT*
CECT 2414	<i>T. reesei</i>	WT	CECT
CECT 2412	<i>Trichoderma koningii</i>	WT	CECT
CECT 2423	<i>T. viride</i>	WT	CECT
CECT 2606	<i>Trichoderma longibrachiatum</i>	WT	CECT
IMI 206040	<i>T. harzianum</i>	WT	IMI†
	<i>H. jecorina</i>	WT	C. P. Kubicek‡
B4	<i>T. harzianum</i>	<i>bgn16.2</i> transformant of <i>T. harzianum</i> CECT 2413	Delgado-Jarana <i>et al.</i> (2000)
13OE1	<i>T. harzianum</i>	<i>papA</i> transformant of <i>T. harzianum</i> CECT 2413	This study
B4OE4	<i>T. harzianum</i>	<i>bgn16.2</i> and <i>papA</i> transformant of <i>T. harzianum</i> CECT 2413	This study

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pepstatin A-sensitive aspartic protease gene of *Trichoderma reesei* resulted in 94% reduction in the total acidic protease activity and increased cellobiase production (Mäntylä *et al.*, 1998).

Occurrence of extracellular proteases in strains of *T. reesei* (Mäntylä *et al.*, 1998) and of *Trichoderma harzianum* (Geremía *et al.*, 1993; Delgado-Jarana *et al.*, 2000) has been reported and several acidic, neutral and basic extracellular proteases have been detected (Delgado-Jarana *et al.*, 2000). Strains of *T. harzianum* are commonly used in biocontrol against fungal plant pathogens (Harman & Björkman, 1998). PRB1, a basic proteinase involved in mycoparasitism has been isolated and the gene cloned (Geremía *et al.*, 1993). Overexpression of the *prb1* gene led to *T. harzianum* strains with improved antagonistic abilities against *Rhizoctonia solani* (Flores *et al.*, 1997). There is no information available about the characteristics of *Trichoderma* neutral proteases or their genes. Some acidic proteases from *T. reesei* have been totally or partially purified (Pitts, 1992; Haab *et al.*, 1990; Dunne, 1982). One of the aspartate proteases from *T. reesei* was stable at pH 3.5 and was insensitive to pepstatin A (Haab *et al.*, 1990), which inhibits most acidic proteases. A second acidic protease, present when *T. harzianum* was grown on cellulose, possessed similar biochemical characteristics but was pepstatin-sensitive (Dunne, 1982). However, sequences of these acidic proteases have not been reported so far. In addition, there are almost no available data on acidic proteases from *T. harzianum*. Two acidic proteases were detected by IEF and zymograms of proteins, obtained after growing *T. harzianum* CECT 2413 in acidic buffered media or in media supplemented with peptidic nitrogen sources (Delgado-Jarana *et al.*,

2000). One of these proteases, which was pepstatin-sensitive, was probably responsible, together with acidic pH, for the degradation of an overexpressed β -1,6-glucanase encoded by *bgn16.2* (Delgado-Jarana *et al.*, 2000).

This study describes the isolation and characterization of a gene from *T. harzianum* CECT 2413, *papA*, that encodes an aspartyl protease. Protease regulation and the effect on the production of β -1,6-glucanase is also discussed.

METHODS

Strains, media and growth conditions. The fungal strains used are described in Table 1. Fungal strains were maintained on potato dextrose agar [2%, w/v, commercial mashed potatoes (dehydrated potato flakes); 2%, w/v, glucose; 2%, w/v, agar]. In liquid cultures, mycelia were grown in 250 ml flasks containing 100 ml salt minimal medium (MM; Penttilä *et al.*, 1987), supplemented with the indicated carbon and nitrogen sources and incubated at 22 °C on a rotary shaker (200 r.p.m.). Two-step cultures were carried out. Mycelia were grown for 40 h in MM supplemented with 2% glucose and 0.5% ammonium sulphate. Mycelia were then washed with distilled water and 2% MgCl₂, resuspended in MM with the appropriate carbon and/or nitrogen sources and incubated for 24 h. When indicated, MM cultures (with glucose and ammonium as carbon and nitrogen sources) were buffered using either 0.2 M sodium citrate pH 3, 0.2 M MES/KOH pH 6 or 0.2 M Tris/HCl pH 8. All media used for transformation and stabilization were as described by Penttilä *et al.* (1987).

DNA procedures and Southern analysis. Standard molecular techniques were performed throughout these studies accord-

Fig. 1. Sequence of the *papA* gene and the deduced protein sequence. The promoter region has been included. Shaded boxes correspond to potential PacC (5'-GCCARG-3'), STRE (5'-CCCT-3') and AreA (5'-GATA-3') binding sites. Sequences marked by a dotted line correspond to putative binding sites for regulatory proteins involved in mycoparasitism (MYC). CAAT and TATA boxes have also been indicated. In the protein sequence, black bars show the ends of the leader and the activation peptides. The active DTG sites of pepsin-like proteases are boxed. Arrows underline the sequences used as primers for PCR amplification. Nucleotide numbering is on the left; amino acid numbering is on the right.

ing to Sambrook *et al.* (1989). DNA isolation and analysis were carried out as described previously (Limón *et al.*, 1995). Southern blot analysis of genomic DNA of *T. harzianum* 2413 and other species of *Trichoderma* (Table 1) was performed using the 900 bp PCR fragment described below as a probe. Genomic DNA was digested with restriction enzymes for which no internal site was present in the amplified fragment, transferred to a nylon membrane and hybridized under low stringency.

RNA procedures and Northern analysis. Mycelia were lysed in a mini-beadbeater (Biospec Products) with 2.3 mm diameter steel beads. RNA was isolated as described by Delgado-Jarana *et al.* (2000). Ten microgrammes of total RNA from each sample were separated in 1.2% agarose/formaldehyde gels, blotted onto nylon membranes and hybridized as described by Sambrook *et al.* (1989). Blots were probed with the complete *papA* gene. Probes were labelled using [β - 32 P]dCTP with the Amersham oligolabelling method (Amersham Pharmacia Biotech). Loading control of Northern blots was checked using radish 18S rDNA.

Isolation of *papA*. *papA* was isolated with primers (Fig. 1) designed by alignment of several fungal acid proteases, carried out using a CLUSTAL W algorithm. Oligonucleotide sequences were 5'-CGACACCGGCTCCGA-3' (direct) and 5'-GGG-

CAACGTCACCAAAGA-3' (reverse). PCR conditions were 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C, then 7 min at 72 °C, using 5 mM MgCl₂. A 900 bp fragment was isolated and used as a probe to screen a genomic library. A 5 kbp *SalI*-*PstI* fragment, which contained an ORF of 1212 bp and 1 kb of a putative promoter region (Fig. 1) was isolated.

Construction of pLMRS3::*papA*. The *papA* ORF was amplified using pSK::*papA* as template DNA and the oligos 5'-CCTTCAAGACAACCACTAGACT-3' and 5'-TCATC-CCTTCAAGATGTCGACCTTC-3'. The PCR reactions were carried out as follows: 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C, using Expand High Fidelity polymerase (Roche). The DNA was treated with *XbaI* and *SalI*, and the fragment was cloned into plasmid pLMRS3 (Mach *et al.*, 1994) already treated with the same enzymes. *papA* is then under the control of the pyruvate kinase gene (*pkI*) promoter from *T. reesei* (Mach *et al.*, 1994).

Transformation procedure. Protoplast preparation, transformation and transformant stabilization were carried out according to Herrera-Estrella *et al.* (1990). *T. harzianum* CECT 2413 wild-type and B4, a transformant strain derived from it which overproduces β -1,6-glucanase (Delgado-Jarana *et al.*, 2000) were cotransformed with plasmids pLMRS3::

papA and pHAT α (Herrera-Estrella *et al.*, 1990): the latter carries the hygromycin resistance gene (*hph*) as a selection marker. Cotransformation was conducted with a 1:10 (*hph*:*papA*) plasmid ratio.

Isolation of extracellular proteins. For protein detection and measurement of enzymic activity, culture supernatants were concentrated by ammonium sulphate precipitation and dialysed against 50 mM potassium acetate buffer, pH 5.5, at 4 °C. Protein concentration was determined by the Lowry method, using BSA as the standard.

Enzyme activity. β -1,6-Glucanase activity was determined by measuring the amount of reducing sugars released from pustulan (Calbiochem) (De la Cruz *et al.*, 1995). The reducing sugar content was determined by the procedure of Somogyi (1952) and Nelson (1957). An enzymic unit was defined as the amount of enzyme that catalyses the release of reducing sugar groups equivalent to 1 μ mol glucose min⁻¹ under the above-described assay conditions. Protease activity was measured by the azocasein procedure of Holwerda & Rogers (1992). An enzymic unit was defined as the amount of enzyme that hydrolyses 1 μ g azocasein (Sigma A-2765) min⁻¹ under the specific assay conditions.

IEF and zymograms. IEF was performed with 10 μ g extracellular proteins in a Multiphor II unit (Amersham Pharmacia). Ampholyte polyacrylamide gels with a pH gradient between 3.5 and 9.5 were used. Zymograms were carried out by overlaying the IEF gel with a 1.5 mm thick 2% agarose gel containing 1% BSA in 50 mM acetate buffer, pH 5.5. Both gels were incubated between two glass plates for 24 h at 37 °C. Proteolytic activities were detected as haloes of BSA degradation.

Western blot analysis. Western blotting was performed using discontinuous SDS–12% PAGE. Prestained molecular mass protein standards (Bio-Rad) were included for molecular mass determination. Transfer onto Immobilon-P membranes (Millipore) was performed using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) following the manufacturer's instructions. Membranes were blocked for 2 h with a solution of 5% skimmed milk in 10 mM Tris/HCl pH 8, 1 mM EDTA, 150 mM NaCl and 0.1% Triton X-100. Filters were then incubated overnight at room temperature with rabbit polyclonal antibody against BGN16.2 (Delgado-Jarana *et al.*, 2000), diluted appropriately in blocking solution, and then washed with blocking buffer. Alkaline phosphatase-conjugated anti-rabbit IgG antibody (Sigma) was used as the secondary antibody.

RESULTS

Isolation of *papA* from *T. harzianum* CECT 2413

Comparison of the amino acid sequences of several fungal proteases from *Aspergillus niger*, *Cryphonectria parasitica*, *T. reesei* and others revealed several conserved regions that were used to design primers for PCR amplification. Not only the conserved domains of the fungal proteases – above all those of *T. reesei* (Mäntylä *et al.*, 1994, 1998) – but the bias introduced by the frequency of codons present in *Trichoderma* genes was taken into account. Oligonucleotides designed corresponded to conserved regions of the fungal proteases and allowed amplification of a 900 bp fragment from *T. harzianum* CECT 2413 genomic DNA. Its identity was

confirmed by sequencing and by comparison with the sequences of known fungal proteases, using a BLASTX algorithm. The 900 bp PCR fragment was used as a probe to screen a genomic library of *T. harzianum* CECT 2413 constructed in λ GEM-11 (Lora *et al.*, 1995). Fig. 1 shows the DNA sequence of the *papA* gene and the hypothetical amino acid sequence of the protease, named PAPA.

Sequence analysis of *papA*

The nucleotide sequence of the ~3 kb cloned fragment revealed that it contains about 1 kb which corresponds to the promoter region, and the entire nucleotide sequence of the *papA* gene (GenBank accession no. AJ276388) (Fig. 1). The 5' non-coding sequence of *papA* was examined for the presence of motifs involved in the regulation of *papA* expression. The sequence, applying the MatInspector algorithm, contains multiple copies of the 5'-GATA-3' motif, which is the recognition sequence of the nitrogen regulatory protein AreA from *Aspergillus* (Ravagnani *et al.*, 1997) and several copies of the 5'-CCCT-3' motif, which corresponds to the recognition sequence of the stress proteins, STRE, involved in gene regulation under stress conditions in *Saccharomyces cerevisiae* (Martínez-Pastor *et al.*, 1996). These motifs have also been found in the basic proteinase (*prb1*) and endochitinase (*ech42*) gene promoter sequences of *T. harzianum* (Cortés *et al.*, 1998). Only one copy of the motif 5'-GCCARG-3', the recognition site for the PacC protein mediating pH regulation in *Aspergillus* (Tilburn *et al.*, 1995), was detected. Putative binding sites for regulatory proteins involved in mycoparasitism (MYC) described in the *prb1* and *ech42* promoter sequences of *T. harzianum* (Cortés *et al.*, 1998), identical to MYC1 (5'-GCTTCA-3') and nearly identical to MYC2 (5'-TTGGCAA-3'), MYC3 (5'-GGGCAC-3') and MYC4 (5'-GGCAWTCGGCAT-3'), and with the same relative position (5'-MYC1 MYC2 MYC3 MYC4-3') were also detected, although their role *in vivo* has still to be demonstrated. In this promoter region there are also TATAA and CAAT motifs, which are related with the initiation of transcription (Tilburn *et al.*, 1995). The sequence prior to the translation initiation triplet is highly conserved, since the TCAA pentamer correlates with the proposed (T/C)CAA(A/C) consensus sequence previous to the AUG codon (Goldman *et al.*, 1998). No polyadenylation signals were detected in the gene sequence.

The *papA* gene seems to consist of an intronless ORF of 1212 bases, which encodes a polypeptide of 404 aa. However the existence of introns of small size that do not alter the reading frame cannot be ruled out. By comparison with the N-terminal region of secreted acid proteases of other filamentous fungi, analysed with the Signal IP v1.1 algorithm, it may be suggested that the PAPA protein is synthesized as zymogen, following the general rules for fungal acid proteases. This region contains an N-terminal preproregion of 52 aa (Fig. 1), formed by a leader peptide of 20 aa, with a potential

Fig. 2. Comparison of the predicted amino acid sequence of *papA* of *T. harzianum* CECT 2413 with the corresponding sequences of the fungal acid proteases CarP from *E. parasitica*, GcSap from *C. gloeosporioides*, PapA from *Podospira anserina*, PepB from *Penicillium janthinellum*, PepA from *A. niger*, PepA from *A. awamori* and AspA from *Penicillium roqueforti*. Dashes indicate gaps introduced by the BLASTX program to optimize the sequence alignments. Amino acids that are conserved in all proteins (or conservative replacements) are shaded in black; those that are conserved in nearly all the proteins are shaded in grey.

proteolytic site between aa 20 (A) and 21 (L) identified with the Signal IPV1.1 algorithm. The leader peptide is followed by an N-subterminal peptide of 32 aa that could be cleaved to activate the enzyme. This peptide ends with the amino acids KR (at positions 51 and 52). A potential protease KEX2 site follows KR (52) in other proteins (Bussey, 1988). This proteolysis would produce a mature active protein. The mature form of the enzyme

would be a 352 residue protein with a calculated molecular mass of 36.7 kDa and a calculated pI of 4.35. There are two DTG motifs for aspartyl proteases. The *papA* sequence also reveals the existence of two lobes, each of which has its own active DTG site (Rawlings & Barret, 1995). No potential post-translational modification signals were detected in the deduced primary sequence.

Fig. 3. Detection of the *papA* gene in different species. Southern blot analysis was carried out with 10 µg genomic DNA digested with *EcoRI* (E) or *PstI* (P). The 900 bp fragment obtained as PCR product was used as probe.

PAPA and homologous proteins found in other organisms

The mature PAPA protein exhibits 43–56% identity with aspartyl proteases of *Colletotrichum gloeosporioides* (54%), *Endothia parasitica* (56%), *Podospora anserina* (48%), *Penicillium roqueforti*, *Penicillium janthinellum* (43–47%), and *Aspergillus oryzae*, *A. niger* and *A. awamori* (44–46%). Alignment of the deduced PAPA amino acid sequence and those aspartyl protease sequences of different filamentous fungal species with which the PAPA sequence displays the highest similarity are shown in Fig. 2. The sequences have been aligned to achieve maximal homology, which corresponds to the amino-terminal region, by using the CLUSTAL W program.

The *papA* gene in *T. harzianum* CECT 2413 and in other *Trichoderma* species

Southern analysis of genomic DNA of *T. harzianum* CECT 2413 and other species of *Trichoderma* (Table 1) showed that the PCR fragment strongly hybridized to a single band in *T. harzianum* CECT 2413 (Fig. 3), indicating that the acid protease activity is encoded by a single gene

in this strain. Southern blot analyses also showed that the *papA* gene was present, and highly conserved, in *Trichoderma viride* CECT 2423. Other bands that hybridized weakly indicated variable degrees of sequence conservation. These sequences were present both among genes of various *Trichoderma* species, including *Hypocrea jecorina*, the sexual form of *T. reesei* (Kuhls *et al.*, 1996) and among other genes (probably protease-encoding genes) present in *T. harzianum* CECT 2413. (Fig. 3). There are protease encoding gene families described in several fungal genera, among them, *Aspergillus* (van den Hombergh *et al.*, 1997a).

Pattern of *papA* mRNA accumulation

Total RNA isolated as described in Methods was hybridized with *papA* (Fig. 4a). No *papA* signal was detected after 24 h incubation in either unbuffered or buffered MM with 2% glucose and 0.5% ammonium, thus indicating that the protease gene is not constitutively expressed. Glucose is probably repressing *papA* gene transcription. A weak signal was observed 24 h after transfer to starvation conditions (MM with 0.1% glucose and 0.5% ammonium), whereas a stronger signal was only shown in those media where organic nitrogen sources, such as yeast extract, casein and, to a lesser extent, peptone, instead of ammonium, had been added (Fig. 4a). Lack of *papA* expression in MM with 2% glucose and 0.5% ammonium, either unbuffered (final pH 2.5) or buffered at pH 3 or 6 indicates that, although *papA* may be regulated by the ambient pH, a specific pH is not sufficient to allow *papA* induction. The effect of carbon and nitrogen sources, as well as that of ambient pH on *papA* expression was more extensively studied by Northern analyses under other conditions (Fig. 4b). In unbuffered media *papA* was strongly expressed only in glucose- and casein-supplemented media. The absence of a nitrogen source does not seem to derepress *papA* gene, whereas substitution of glucose by glycerol, in media with either ammonium or casein does not derepress *papA* either. *papA* expression does therefore seem to be weakly controlled by glucose repression. Expression of *papA* was strongly repressed by the simultaneous presence of ammonium and glucose or glycerol. In MM buffered at pH 3 or 8 no expression of *papA* was observed, when either ammonium or casein was used as the nitrogen source, indicating that pH regulation overrides induction by casein, but that pH alone is not sufficient to induce *papA* expression under conditions of nitrogen repression. Results were identical when Northern experiments were carried out with mRNA obtained from *T. harzianum* CECT 2413 wild-type (Fig. 4a, b) or from strain B4 which overproduces β -1,6-glucanase (Delgado-Jarana *et al.*, 2000) (data not shown).

Isolation of *papA* transformants

T. harzianum CECT 2413, wild-type and B4 strains were cotransformed with the plasmids pLMRS3::*papA*

Fig. 4. Northern blots carried out with total RNA obtained from mycelia of *T. harzianum* CECT 2413 grown for 40 h in MM (2% glucose and 0.5% ammonium) and transferred and incubated for 24 h in MM and the indicated conditions. The 900 bp PCR product (a, b) or the complete *papA* ORF (c) were used as probes for *papA* mRNA. Radish 18S rDNA was used as loading control. (a) Nitrogen sources (0.5%): ammonium (Am), yeast extract (YE), peptone (Pep) or casein (Cas). Lanes 1–5, unbuffered media; lanes 6 and 7, pH 3 and pH 6 buffered media respectively. (b) Lanes 1–6, unbuffered media; lanes 7 and 9, pH 3 buffered medium; lanes 8 and 10, pH 8 buffered media. + Presence and – absence of the indicated carbon or nitrogen sources. (c) Lanes 1–4, MM with 2% glucose and 0.5% ammonium; lanes 5–8, MM with 2% glucose and 1% yeast extract. The strains are described in Table 1.

and pHAT α . Two stable transformants: 130E1, derived from *T. harzianum* CECT 2413 (wild-type), and B4OE4, derived from strain B4, were chosen for further studies. Both strains contained additional copies of the *papA* gene. Integration of *papA* had occurred ectopically in tandem or as single copy in several places in the genome (data not shown), as occurred with the *bgn16.2* gene (Delgado-Jarana *et al.*, 2000).

Patterns of *papA* mRNA accumulation and of protease and β -1,6-glucanase activities

The strains were cultivated in MM with 2% glucose as the carbon source and either 0.5% ammonium or 1% yeast extract as the nitrogen source, as described in Methods. The mRNA level of *papA* was similar in the transformants 130E1, which overexpressed only *papA*,

Table 2. Protease and β -1,6-glucanase activities of *T. harzianum* CECT 2413 and strains B4, 13OE1 and B4OE4, cultivated in MM with 2 % glucose and either ammonium or yeast extract as the nitrogen source

Experiments were repeated at least twice and assays were carried out in triplicate. Representative results of one experiment are shown. ND: Not detected.

Strain	Ammonium		Yeast extract	
	Protease (U g ⁻¹)	β -1,6-Glucanase (U g ⁻¹)	Protease (U g ⁻¹)	β -1,6-Glucanase (U g ⁻¹)
WT	2.36 $\pm$ 0.5	ND	10.9 $\pm$ 1.3	ND
B4	3.66 $\pm$ 1.6	ND	11.7 $\pm$ 0.2	124.6 $\pm$ 7.4
13OE1	2.66 $\pm$ 0.6	ND	44.6 $\pm$ 1.4	1.1 $\pm$ 0.1
B4OE4	3.28 $\pm$ 1.0	ND	47.5 $\pm$ 1.7	185.5 $\pm$ 25

Fig. 5. Extracellular proteins obtained from supernatants of *T. harzianum* cultivated in unbuffered MM with 2 % glucose and either 0.5 % ammonium (0.5 % ammonium) (final pH 2.5) or 1 % yeast extract (1 % YE) (final pH 4.1). Ammonium-precipitated extracellular proteins were separated in SDS-12 % PAGE and (a) stained with Coomassie Brilliant Blue R-250 or (b) subjected to Western blot experiments with anti-BGN16.2 antibodies. The strains are described in Table 1.

and B4OE4, which overexpressed both *papA* and *bgn16.2* (Fig. 4c). Data on protease activity, measured in the supernatants of the cultures of the four strains, indicated that in MM with ammonium, *papA* single transformant 13OE1 or the double transformant B4OE4 had similar values, and were also similar to those of the wild-type and strain B4. Protease values increased about 15-fold in supernatants from *papA* transformants and 3–4-fold in supernatants from the wild-type and B4 strain, when they were cultivated in medium with yeast extract compared to medium with ammonium (Table 2). Extracellular proteins were obtained from the supernatants of MM with either ammonium or yeast extract by ammonium precipitation; the proteins were separated by SDS–12 % PAGE and were stained with Coomassie Brilliant Blue R-250. PAPA and/or BGN16.2 proteins were observed only in supernatants from cultures with yeast extract (Fig. 5a). A zymogram of extracellular proteins from MM with yeast extract is presented in Fig. 6. There is a single protein with

protease activity which was overexpressed in the *papA* transformant 13OE1, which possesses a pI of 4.5. Protease inhibition was obtained when pepstatin, an aspartyl protease inhibitor, was used; no inhibition was observed when PMSF was used instead.

Western blot experiments using anti-BGN16.2 antibodies were also carried out (Fig. 5b). In all cases, increased β -1,6-glucanase activity (strains B4 and B4OE4 cultivated in MM with yeast extract) correlated with higher levels of BGN16.2 protein (Fig. 5b). Neither BGN16.2 protein (Fig. 5b) nor activity (Table 2) was detected in the cultures of any of the strains grown in MM with glucose and ammonium. In yeast extract both β -1,6-glucanase activity (Table 2) and protein (Fig. 5a) were detected either in the presence or in the absence of the PAPA protein. This result indicates that PAPA does not seem to be responsible for the proteolysis of β -1,6-glucanase which occurs in unbuffered MM with glucose and ammonium (Table 2 and Fig. 5), although the β -1,6-

Fig. 6. IEF and zymogram of extracellular proteins obtained after growing *T. harzianum* CECT 2413 wild-type (WT) and *papA* transformant 13OE1 in MM with 2% glucose and 1% yeast extract. Concentrated supernatants from 13OE1 were incubated with 1 μ M pepstatin (+peps) or 0.1 mM PMSF (+PMSF) before IEF separation and zymogram, which was carried out to determine protease activity.

glucanase enzyme displayed some instability at pHs below 7 (Delgado-Jarana *et al.*, 2000) (Fig. 5b).

DISCUSSION

The isolation, sequence and regulation pattern of a gene from *T. harzianum* that encodes an acidic protease, *papA*, is described in this study for the first time. IEF and zymograms had previously allowed the identification of acidic, neutral and basic extracellular proteases in *T. harzianum* CECT 2413 (Delgado-Jarana *et al.*, 2000).

In a different strain of *T. harzianum*, a basic protease of 31 kDa, PBR1 (Geremía *et al.*, 1993), probably equivalent to the basic proteinase detected in *T. harzianum* CECT 2413 (Delgado-Jarana *et al.*, 2000), was identified as a subtilisin which was induced by fungal cell walls and played a fundamental role in mycoparasitism against phytopathogenic fungi (Flores *et al.*, 1997) and biocontrol of root-knot nematodes (Sharon *et al.*, 2001). The role of other fungal basic proteases such as those of *Metarhizium anisopliae*, *Bauveria bassiana* (St Leger *et al.*, 1997) or *Sclerotinia sclerotiorum* (Poussereau *et al.*, 2001) has also been regarded as highly significant during antagonistic interactions with other organisms, and their regulation seems to depend on cell wall components of the antagonized hosts.

With regard to acidic proteases, several pepsin-like aspartyl proteases have been described in *T. reesei*

(Dunne, 1982; Haab *et al.*, 1990) and *T. harzianum* (Delgado-Jarana *et al.*, 2000) with pIs that varied from 4.3 to 4.9. None of them have been reported to be completely purified, with the exception of a trypsin-like protease (Suárez *et al.*, 2000) and the aspartyl protease called trichodermapepsin, which has been purified and cloned (Pitts, 1992; Mäntylä *et al.*, 1994). Some of these acidic proteases are pepstatin-inhibitable, but others are insensitive to this inhibitor (Haab *et al.*, 1990) and they have been detected in casein- or cellulose-supplemented media. The pepsin-like aspartyl protease gene from *T. harzianum* CECT 2413, *papA*, isolated and characterized in this study, possesses characteristics that are similar to those of the trichodermapepsin of *T. reesei* (Mäntylä *et al.*, 1994) (Fig. 1). The PAPA protein, which has been only partly purified, showed inhibition by pepstatin when it was overproduced (Fig. 6).

In this study, glucose represses *papA* transcription. Substitution of glucose by glycerol in media with either ammonium or casein does not derepress *papA*. By contrast, other protease genes such as *acp1* from *S. sclerotiorum* are strongly expressed when glycerol – considered a neutral carbon source – is added to the medium instead of glucose (Poussereau *et al.*, 2001). In addition, *papA* was regulated by at least the nitrogen and carbon sources and by ambient pH (Fig. 4). The results are consistent with the presence of potential sites for nitrogen (AreA) and pH (PacC) control described in *Aspergillus* and other filamentous fungi (Espeso *et al.*, 1993) that are also found in the *papA* promoter, but the lack of potential CreA sites suggests carbon regulation may take place by a system different from CreA, as has been suggested in other fungi (Espeso *et al.*, 1993; Ronne, 1995). Other protease genes are also repressed by glucose and ammonia, such as *pepA* from *A. niger* (Berka *et al.*, 1990), and/or induced by peptidic nitrogen sources (casein), such as the *aspA* protease gene from *Penicillium roqueforti* (Gente *et al.*, 1997). In this study basic and acidic pHs completely repressed *papA* gene expression, even in the presence of casein (Fig. 4). A similar phenomenon at alkaline pH has been described for *aspA* from *P. roqueforti* (Gente *et al.*, 2001). At low pH the PAPA protease might be denatured and/or pH cleaved as occurred to the β -1,6-glucanase (Delgado-Jarana *et al.*, 2000). At alkaline pH *papA* product might not be processed into its mature, active form (Gente *et al.*, 2001). Those peptides which should activate *papA* are not liberated from casein.

Overexpression of PAPA did not affect cell viability, and in *papA* transformants with multiple copies of the gene, the *papA* gene product could be detected in yeast extract (Figs 5 and 6) but not in MM minimal medium with 2% glucose and 0.5% ammonium. Overexpressed PAPA coexisted with overexpressed β -1,6-glucanase and both proteins were only present in media with organic nitrogen sources such as yeast extract (Figs 5 and 6). The absence of PAPA in MM minimal medium with glucose and ammonium probably resulted from proteolysis and denaturation due to a double effect of aspartyl proteases and extreme pHs, as occurred with BGN16.2 (Delgado-

Jarana *et al.*, 2000). PAPA is detected at higher pHs and when alternative protease substrates (i.e. yeast extract) are present in the medium. Peptidic nitrogen sources are frequently added during protein overproduction to avoid proteolysis of the desired protein (Jeenes *et al.*, 1991). Lack of PAPA when BGN16.2 is proteolysed (Figs 5 and 6) indicated that this protease is not responsible for BGN16.2 degradation.

Disruption of *papA* would indicate whether or not PAPA plays a role in β -1,6-glucanase degradation. However, attempts to disrupt the protease gene in the wild-type and the B4 transformant have been unsuccessful. The hygromycin resistance gene *hph* that was used as the selection marker was flanked at both ends by 1.4 kb fragments of the *papA* gene. In all cases the transformants isolated possessed several ectopic copies of the *hph* gene but maintained unaltered the endogenous *papA* gene. The reason for the lack of *papA* gene disruptants could be the small size of the homologous fragments rather than *papA* being essential. As occurs in other filamentous fungi (Asch & Kinsey, 1990) *T. harzianum* CECT 2413 is probably very reluctant to undergo homologous recombination, which does not take place unless the homologous region possesses a minimal size.

Acidic proteases seem to be responsible for degradation of extracellular proteins such as chitinases and glucanases, cellulases and other homologous or heterologous proteins overproduced for different applied purposes (Margolles-Clark *et al.*, 1996; Delgado-Jarana *et al.*, 2000; Hagspiel *et al.*, 1989; Nyssönen *et al.*, 1993). In this study, overexpression of PAPA did not result in degradation of overproduced β -1,6-glucanase. Rather, a 30% increase in β -1,6-glucanase activity was detected in double *bgn16.2 papA* transformants, compared to single *bgn16.2* transformants (Table 2). This increase could result from a dilution effect due to the BGN16.2 and PAPA proteins being the targets of the same proteases, although the reason is not yet known for sure.

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