Appl Microbiol Biotechnol (2004) 65: 46–55
DOI 10.1007/s00253-004-1610-x

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Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity

Received: 4 December 2003 / Revised: 1 March 2004 / Accepted: 5 March 2004 / Published online: 20 May 2004

Abstract Mycoparasitic *Trichoderma* strains secrete a complex set of hydrolytic enzymes under conditions related to antagonism. Several proteins with proteolytic activity were detected in culture filtrates from *T. harzianum* CECT 2413 grown in fungal cell walls or chitin and the protein responsible for the main activity (PRA1) was purified to homogeneity. The enzyme was monomeric, its estimated molecular mass was 28 kDa (SDS-PAGE), and its isoelectric point 4.7–4.9. The substrate specificity and inhibition profile of the enzyme correspond to a serine-protease with trypsin activity. Synthetic oligonucleotide primers based on N-terminal and internal sequences of the protein were designed to clone a full cDNA corresponding to PRA1. The protein sequence showed <43% identity to mammal trypsins and 47–57% to other fungal trypsin-like proteins described thus far. Northern analysis indicated that PRA1 is induced by conditions simulating antagonism, is subject to nitrogen and carbon derepression, and is affected by pH in the culture media. The number of hatched eggs of the root-knot nematode *Meloidogyne incognita* was significantly reduced after incubation with pure PRA1 preparations. This nematicidal effect was improved using fungal culture filtrates, suggesting that PRA1 has additive or synergistic effects with other proteins produced during the antagonistic activity of *T. harzianum* CECT 2413. A role for PRA1 in the protection of plants against pests and pathogens provided by *T. harzianum* CECT 2413 is proposed.

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Introduction

Fungal strains grouped in the genus *Trichoderma* possess a wide spectrum of evolutionary responses that range from very effective soil colonization, with high biodegradation potential, to non-strict plant symbiosis by strains colonizing the rhizosphere. In addition, some groups of strains within this conglomerate of biotypes are able to antagonize phytopathogenic fungi by using substrate colonization, antibiosis, and mycoparasitism as the main mechanisms (Hjeljord and Tronsmo 1998). This antagonistic potential is the base for effective applications of *Trichoderma* strains as alternatives to the chemical control of a wide set of phytopathogenic fungi (Harman and Björkman 1998). *Trichoderma* spp. have also been described as biocontrol agents against plant-parasitic nematodes. Several reports showed that *Trichoderma* spp. are able to suppress *Meloidogyne* spp. populations (one of the most economically important group of plant-parasitic nematodes worldwide) and increase crop yields (Rao et al. 1996; Sharon et al. 2001; Spiegel and Chet 1998; Windham et al. 1993). Although the information about the mechanisms of this fungal activity against root-knot nematodes is limited, the ability of *T. harzianum* Rifai to colonize eggs and infect second-stage juveniles (J2) in vitro has been demonstrated (Saifullah and Thomas 1996; Sharon et al. 2001).

The strong biodegradation and substrate-colonization properties of many *Trichoderma* strains are the result of an amazing metabolic versatility and a high secretory poten-

tial that lead to the production of diversified sets of hydrolytic enzymes. Among others, cellulases and xylanases, involved in plant-material degradation, have been described as components of the multi-enzymatic system (Biely and Tenkanen 1998). Similarly, the mycoparasitic process is based on the secretion of complex cocktails of enzymes involved in penetration of the host cell wall (see references Benitez et al. 1998 and Lorito 1998 for reviews). Some of these enzymes display antifungal activities when applied in vitro, either alone or combined, against plant pathogens. A principal role in mycoparasitism has been attributed to chitinases and glucanases. However, fungal proteases may be significantly involved in antagonistic activity, not only in the breakdown of the host cell wall (composed of chitin and glucan polymers embedded in, and covalently linked to, a protein matrix; Kapteyn et al. 1996), but also by acting as proteolytic inactivators of pathogen enzymes involved in the plant infection process (Elad and Kapat 1999).

Extracellular proteolytic activities in *Trichoderma* species have long been recognized and they have been attributed to antagonistic and biocontrol activities (Bertagnolli et al. 1996; De Marco and Felix 2002; Elad and Kapat 1999; Rodriguez Kabana et al. 1978). Nonetheless, the *Trichoderma* spp. proteolytic system involved in antagonism has not been explored in depth and only a subtilisin-like protease (PRB1) from *Trichoderma atroviride* has been extensively studied and the gene (*prb*1) cloned (Geremia et al. 1993). This gene is expressed during mycoparasitic interactions, in the presence of cell walls or chitin (Cortes et al. 1998; Olmedo-Monfil et al. 2002), and its overexpression improves biocontrol activities of transformed *T. atroviride* strains against the fungus *Rhizoctonia solani* (Flores et al. 1997). In addition, the protease encoded by *prb*1 also appears to participate in virulence against the nematode *Meloidogyne javanica* (Sharon et al. 2001). The importance of proteolytic activity in biocontrol processes has been reported for other fungi, e.g. the nematophagus fungi *Pochonia chlamydosporia* (*Verticillium chlamydosporium*) (Segers et al. 1994) and *Arthrobotrys oligospora* (Ahman et al. 2002), and in entomopathogenic fungi, such as *Metarhizium anisopliae* (St. Leger et al. 1995) and *Beauveria bassiana* (Urtz and Rice 2000).

In this report, we describe the purification, biochemical characterization, and cDNA cloning of a trypsin-like protease, PRA1, from the biocontrol agent *Trichoderma harzianum* CECT 2413. The enzyme is able to degrade fungal cell wall proteins, and its regulation supports its involvement in antagonistic processes. Furthermore, the activity of pure protein preparations against hatching eggs of *Meloidogyne incognita* is the first report of a direct effect on nematodes for an isolated gene product from *Trichoderma* spp. This explains the wide antagonistic activity of enzymatic cocktails secreted by *Trichoderma* strains, which is not limited to phytopathogenic fungi, and suggests interesting agricultural applications for this protease.

Materials and methods

Organism and culture conditions

T. harzianum CECT 2413 was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain), and was maintained in tubes with potato dextrose agar (Sigma). Extracellular proteins were produced in MM medium (Penttilä et al. 1987) under two-step culture conditions to reduce the dependence on total growth as described (Ait-Lahsen et al. 2001). Fungal cell walls from the strawberry pathogen *Colletotrichum acutatum* IMI 364856 (CABI Bioscience, Egham, UK) and *Botrytis cinerea* CECT 2100 were prepared as previously reported (Fleet and Phaff 1974). For Northern analysis, mycelia were collected by filtration, thoroughly washed with sterile water, lyophilized and kept at −80°C until RNA extraction. Nitrogen starvation conditions corresponded to a 100-fold decrease in the concentration of ammonium sulfate in the medium (50 mg/l). When indicated, media were buffered using MES/KOH 0.2 M (pH 6.5), or HCl (pH 2.5). To obtain mycelium for Southern analysis *T. harzianum* was cultured for 4 days on potato dextrose broth (Sigma).

Preparation and analysis of culture filtrates

After removing mycelium by filtration, proteins contained in the culture filtrates were concentrated and treated as described (De la Cruz et al. 1992). The amount of protein was determined by the Bio-Rad assay (Bradford 1976). Enzymes with proteolytic activity were detected by SDS-PAGE with 0.06% casein (Merck) incorporated in the gel (Garcia-Carreño et al. 1993). Proteolytic activity was visualized as clear zones on a blue background after Coomassie blue staining.

Chromatofocusing and gel-filtration chromatography

The concentrated (75×) culture supernatant from *T. harzianum* was loaded onto a polybuffer Exchanger PBE 94 column equilibrated with 25 mM imidazol/HCl buffer, pH 7.2. Proteins were eluted at a flow rate of 10 ml/h (0.2 ml each fraction) with Polybuffer 74/HCl: water (1:8), pH 4.0. The subsequent FPLC gel filtration was carried out with a Protein Pack 125 column (Pharmacia) equilibrated in 50 mM sodium acetate buffer, pH 5.5, and proteins were eluted with the same buffer at a flow rate of 12 ml/h (0.2 ml each fraction). Chromatofocusing and gel filtration were monitored for protein by measuring the absorbance at 280 nm, and for enzyme activity by measuring hydrolysis of azocasein (Sigma) (Holwerda and Rogers 1992).

Enzymatic assays

Endopeptidase activity against *p*-nitroanilide substrates (Sigma) was measured in reactions of 100 µl of 100 mM sodium phosphate buffer, pH 7.5, containing 1 mM of substrate. After 20 min of incubation at 30°C, the reaction was stopped with 50 µl of 2% acetic acid. *p*-nitroaniline (*p*-NA) released was measured in 100-µl aliquots in microtiter plates at 405 nm. One unit of activity represented the hydrolysis of 1 nmol *p*-NA/min under the assay conditions. Inhibition studies and the effect of temperature (from 30 to 85°C) and pH (from 3 to 9) on the activity of purified protease PRA1 were carried out with 75 ng of the protease and *N*-acetyl-Ile-Glu-Ala-Arg-pNA as substrate. Protease preparations were incubated with inhibitors at 30°C for 30 min before developing the assay. Residual activity was determined as a percentage of the activity in control samples without inhibitor. Kinetic constants were measured with 25 ng of the purified PRA1 in 1 ml of buffer at different substrate concentrations (0.05–2 mM). *p*-NA appearance

was monitored for 10 min at 405 nm. K_m, K_{cat} and K_{cat}/K_m were determined from the initial rates of hydrolysis by using Lineweaver–Burk plots.

Proteolytic activity of PRA1 against *C. acutatum* cell walls was assayed in reaction mixtures containing 1 μg enzyme and 1% fungal cell walls in 600 μl of 100 mM sodium acetate buffer, pH 5.5. The mixture was incubated on a shaker at 30°C for 9–24 h, and the reaction was stopped by centrifugation. The supernatant was filtered through a 0.45-μm filter and the release of soluble amino groups from cell walls was determined in the supernatant by a ninhydrin assay (Lee and Takahashi 1966). Enzyme activity was expressed as μg L-leucine equivalents. Control reactions contained fungal cell walls without enzyme and enzyme without substrate.

Antifungal assay

Antifungal activity was determined as described previously (Ait-Lahsen et al. 2001) using microwell plates and estimating the effect on germination and hyphal growth of *C. acutatum*. Up to 1,000 ppm of purified PRA1 were tested, and positive controls were run using crude culture filtrates from *T. harzianum* CECT 2413 grown on fungal cell walls.

PRA1 microsequencing and DNA and RNA manipulations

Amino-terminal and internal peptide sequencing was performed by Eurosequence b.v. (Groningen, Holland). Fungal total DNA was isolated according to the protocol previously described (Reader and Broda 1985). Fungal RNA was isolated using TRIZOL reagent (Life Technologies), according to the manufacturer's instructions. Southern blotting and Northern blotting were done using standard techniques (Sambrook et al. 1989). DNA sequencing was carried out by the Sanger method (Sanger et al. 1977).

Construction of a cDNA library and identification of *pra*1 cDNA clones

A cDNA library was constructed using the Uni-Zap XR vector and Gigapack III Gold Packaging Extract (Stratagene) from poly(A)$^+$ RNA isolated from mycelium of *T. harzianum* CECT 2413 (incubated for 9 h in MM with 0.5% of fungal cell walls). After the excision process, plasmidic DNA was isolated from the amplified library and used as a template for PCR with oligonucleotides designed from peptide sequencing data. PCR conditions were: 95°C, 1 min; 55°C, 1 min; 72°C, 1 min, repeated 35 times. Approximately 2×10^5 PFU from the amplified cDNA library were screened by plaque hybridization, according to Stratagene's protocol. Plaques that gave a strong hybridization signal were purified and analyzed.

Phylogenetic data analysis

Amino acid sequences of PRA1 and 18 trypsins available in the EMBL (European Molecular Biology Laboratory, Heidelberg, Germany) database were aligned using CLUSTALX 1.81 (Thompson et al. 1997). Phylogenetic analysis was carried out with PAUP program (Phylogenetic Analysis Using Parsimony, Version 4.0, Sinauer Associates, Sunderland, Mass., USA), and a neighbor-joining tree was constructed using the Kimura-2-parameter distance model (Kimura 1980). Confidence values were assessed from 500 bootstrap replicates of the original data. The tree was rooted by designating the amino acid sequence of chymotrypsin CHY1 from *Streptomyces glaucescens* as outgroup.

M. incognita egg hatch assays

The nematode population was obtained and maintained as described (Nico et al. 2002). The effect of protease PRA1 from *T. harzianum* CECT 2413 on egg hatch of *M. incognita* was investigated by in vitro assays, as follows: approximately 200 eggs were added to solutions of purified protease PRA1, boiled PRA1, trypsin-bovine (Sigma), crude culture filtrates from *T. harzianum* CECT 2413 grown in *C. acutatum* cell walls, or sterile deionized distilled water (SDDW). Each treatment was tested in a separate well of 24-well titration plates. The plates were incubated at 25 (±1°C) in the dark and gently shaken every 12 h. Numbers of J2s that emerged were recorded at 2- to 3-day intervals for 25 days of incubation. At the end of the experiment, eggs and J2s from each well were counted, and the numbers of hatched J2s were expressed as a cumulative percentage of viable J2s. Treatments were replicated six times and the experiment was repeated twice. The area under cumulative percentage hatch (AUCPH) was estimated by trapezoidal integration (Campbell and Madden 1990). AUCPH and the final cumulative percentage hatch were analyzed by ANOVA. Treatment means of AUCPH and final cumulative egg hatch at each treatment were compared using Fisher's protected least-significant-difference test (LSD) at P=0.05 (Gomez and Gomez 1984). Data were analyzed using Statistix (NH Analytical Software, Roseville, MN).

Results

Identification and purification of PRA1

When *T. harzianum* CECT 2413 was grown in MM supplemented with cell walls of *C. acutatum* (0.5%) as carbon source, at least three extracellular proteases could be detected in culture filtrates using SDS-PAGE protease assays. The major activity band was observed at 9 h and became more intense after longer culture times. The molecular mass of this protease (28 kDa) was lower than that of protease PRB1 (31 kDa), previously described in *T. atroviride* IMI 206040 (Geremia et al. 1993). Similar results were obtained when chitin (Sigma) (1%) was used as carbon source. In culture filtrates supplemented with glucose (2%) as carbon source, only a very weak band of activity, close to 31 kDa, was detected at 48 h.

The major protease detected was purified from 48-h cell-wall-containing culture filtrates and subsequently characterized. Purification was carried out by ammonium sulfate precipitation at 90% saturation, acidic chromatofocusing (pH 7.2–4), and gel-filtration chromatography. During chromatofocusing, two peaks with proteolytic activity were detected in the eluted fractions (Fig. 1a). The fractions corresponding to the acidic peak (pH 4.9–4.7) displaying protease activity were analyzed by casein-SDS-PAGE, and the 28-kDa protein was identified as being responsible for the proteolytic hydrolysis. Because of its acidic pI (4.8), this protein was named PRA1 (protease, acidic, 1), in order to distinguish it from the basic protease PRB1 (pI 9.2) previously characterized in *Trichoderma* (Geremia et al. 1993). The fractions within the acidic peak were pooled, concentrated with10-kDa Centricon devices, and subjected to FPLC gel filtration. Active fractions containing PRA1 were pooled and concentrated again. Purification factors and yields at each step are summarized in Table 1. As can be seen in Fig. 1b, the final preparation

migrated as a single band of protein and activity on SDS-PAGE and casein-SDS-PAGE, respectively, indicating a homogeneous protein with an apparent molecular mass of 28 kDa. When 2-mercaptoethanol was added to the sample buffer, no changes were observed in the molecular mass, although activity of the protein was not recovered under these conditions (data not shown).

Peptide sequencing of purified preparations of PRA1 was carried out with the double aim of comparing the sequences with those contained in the databases, and designing degenerate oligonucleotides to clone the gene coding for the protein. The sequences obtained from the N-terminal (IVGGTTAALGEFP) and internal peptide (DSXSGDSGGPIIDPSG) of the protein showed similarity (60–85% identity) to serine-peptidases belonging to the chymotrypsin family S1, including the recently published N-terminal peptide of a peptidase from *Trichoderma viride* (Uchikoba et al. 2001).

Enzymatic characterization

The catalytic mechanism of PRA1 was determined with the use of standard inhibitors. Enzymatic activity was strongly inhibited by 1 mM PMSF (78% inhibition), indicating that PRA1 belongs to the serine-type peptidase group. As expected, the aspartic-peptidase, cysteine-peptidase and metallo-peptidase inhibitors (0.1 mM pepstatin, 1 mM iodoacetamide, and 1 mM EDTA, respectively) had a weak effect on the protease, with less than 11% inhibition.

PRA1 substrate specificity was assayed using synthetic substrates for proteases belonging to the chymotrypsin family. The peptide *N*-acetyl-Ile-Glu-Ala-Arg-pNA (specific for trypsins) proved to be an excellent substrate for PRA1, with an estimated K_m of 0.22 mM, K_{cat} of 39.6 s^{-1} and K_{cat}/K_m of 180.2 mM^{-1} s^{-1}. In contrast, PRA1 showed very weak activity towards chymotrypsins substrate (*N*-succinyl-Ala-Ala-Pro-Phe-pNA), and was unable to hydrolyze substrate for elastases (*N*-succinyl-Ala-Ala-Pro-Leu-pNA). These results indicate the preference of PRA1 for a polar residue (Arg) at the carboxyl side of the cleaved bond, as expected for a trypsin-like protease.

PRA1 was found to have an optimum temperature close to 35°C. Only 16% of the activity remained at 45°C, and minimal activity was detected above 50°C. The optimum pH was in the range of 7–8, and the activity declined above and below this pH interval.

PRA1 displayed protease activity when it was incubated with *C. acutatum* cell walls. In addition, adsorption experiments to *C. acutatum* and *B. cinerea* cell walls showed that PRA1 displays affinity for these structures (data not shown). The antifungal activity against *C. acutatum* of PRA1 was evaluated in microculture assays according to the method of Ait-Lahsen et al. (2001). No effect on spore germination or hyphal growth was detected when pure PRA1 preparations were added to the microcultures.

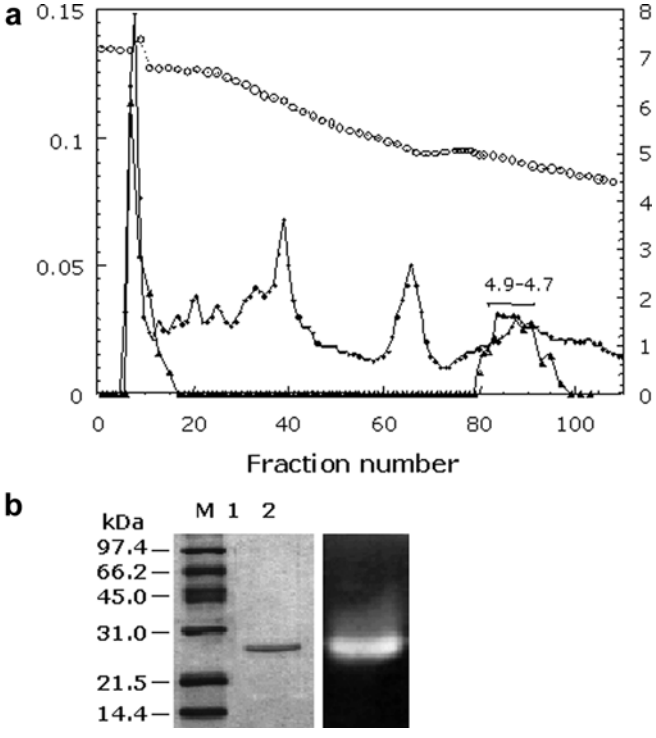

Fig. 1 **a** Preparative chromatofocusing of a concentrated culture filtrate from *Trichoderma harziamum* CECT 2413: protein at A_{280} nm (*filled circles*); protease activity (U/ml) (*filled triangles*); pH (*open circles*). **b** SDS-PAGE and casein-SDS-PAGE (*lanes 1 and 2*, respectively) of purified protease PRA1 (0.5 μg). *Lane M* Bio-Rad low range standards (sizes are indicated on the left)

Molecular cloning and sequencing of *pra*1 full-length cDNA

Based on the sequence of the N-terminal and an internal peptide of the protein, degenerate oligonucleotides were designed (sense: ACTGCIGC(G/T)TTIGGIGA(A/G)TT(T/C)CC-3′; antisense: 5′-GGGTCTAT(T/G)ATIG-GIC-CICC-3′) to clone *pra*1 by PCR. Under the conditions described in "Material and methods", a single fragment (557-bp) was repetitively amplified and further subcloned into pGEM-T and sequenced (data not shown). BLASTX analysis of the sequence showed a high degree of similarity with trypsin-like proteases. To obtain a complete cDNA, the PCR product was used as a probe to screen the mentioned cDNA library. Several positive clones were detected and isolated, and the one containing the largest insert (954-bp fragment) was chosen for further characterization. The full-length sequence and deduced amino acids are shown in Fig. 2. The sequence immediate to the first ATG of the transcript includes nucleotides conserved in most of the genes from *Trichoderma* spp. (Goldman et al. 1998). The open reading frame contained between this ATG and the first termination codon (TAA) is 774 bp long, and encodes a protein of 258 amino acids (25,784 Da). The predicted protein contains all the peptides sequenced from PRA1, demonstrating that the cloned cDNA codes for this protein. Southern analysis of genomic DNA from

Table 1 Purification of protease PRA1 from *Trichoderma harzianum*

Step	Volume (ml)	Total protein[a] (mg)	Yield (%)	Total activity[b] (U)	Specific activity (U/mg)	Purification (fold)
$(NH_4)_2SO_4$ precipitation	2	9.23	100	59.3	6.42	1.0
Cromatofocusing eluate	0.54	0.83	21	12.5	15.0	2.3
Gel filtration eluate	0.83	0.088	12	6.9	78.4	12.2

[a]The amount of protein was determined by the Bio-Rad assay.
[b]Protease activity was measured on azocasein.

T. harzianum CECT 2413 indicated that PRA1 is encoded by a single gene. However, use of low-stringency conditions revealed several additional bands, indicating the presence of other related sequences. The gene was also present in other *Trichoderma* species and haplotypes (data not shown).

PRA1 protein sequence: comparison to other proteins

*pra*1 is predicted to encode a mature protein of 229 amino acids and a molecular mass of 25.023 kDa, which is slightly lower than the size estimated by SDS-PAGE (28 kDa). Three possible O-glycosylation sites (S-T) can be identified at amino acids S^{48}, S^{132} and S^{169}. However, the protein lacks sites for N-glycosiyation (NKT/S). The calculated pI (4.91) for mature protease is in good accordance with that estimated by preparative chromatofocusing (4.7–4.9). Furthermore, comparison of the deduced amino acid sequence with the experimentally determined N-terminal sequence of PRA1 revealed that the mature protein starts at residue 30. A signal-sequence cleavage site prediction, carried out according to empirical rules (Nielsen et al. 1997), suggests that the cleavage occurs between G^{20} and A^{21}, indicating a putative signal peptide of 20 amino acids (Fig. 2), including a secondary α-helix structure, a core of hydrophobic residues, and a helix-breaking residue (P^{15}). The remaining sequence between the signal peptide and mature protease (A^{21}–D^{29}) could therefore correspond to the propeptide-region found in many proteases (Markaryan et al. 1996).

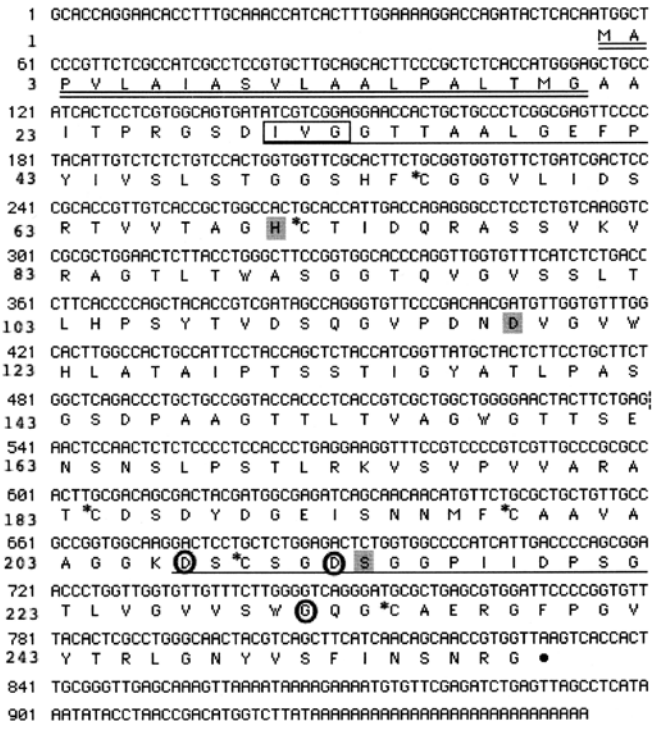

Fig. 2 Nucleotide and predicted amino acid sequences of *pra*1 cDNA. The N-terminal and internal peptide sequences obtained directly from the pure protein are *underlined*. The putative signal peptide is *double underlined*. Mature PRA1 starts at the boxed sequence IVG. Putative amino acids at the active site are marked by *gray background shading*. Other putative amino acids that are essential for assembly of catalytic sites, or substrate specificity of trypsins (Perona et al. 1995) are *circled*. Cysteine residues participating in disulfide bonds in members of the chymotrypsin family are marked by *asterisks*

The complete amino-acid sequence of PRA1 showed a consistent degree of similarity with proteases from a variety of organisms. The highest identities (47–57%) were found with trypsin-like proteases previously described in filamentous fungi. Lower identity percentages were found with trypsins from invertebrates (<45%), mammalians (<43%) and bacteria (<39%). The alignment of predicted sequence of PRA1 with these fungal proteases using CLUSTAL (Higgins and Sharp 1988) allowed identification of the catalytic triad (H^{70}, D^{118} and S^{213}) and conserved sequences around both the active serine (DSCSGD*S*GGPII) and the catalytic histidine (VTAG*H*C), confirming that PRA1 belongs to the chymotrypsin family S1 (Fig. 2).

Phylogenetic analysis of the amino acid sequences of PRA1 and representative trypsins from other organisms provides strong statistical support (100%) for the separation of vertebrate, invertebrate, fungal and bacterial proteases into independent clusters (Fig. 3). PRA1 appears in the same cluster as other fungal trypsin-like proteases, such as TRY1 and TRY2 from *M. anisopliae* (Smithson et al. 1995), and ALP1 from *Cochliobolus carbonum* (Murphy and Walton 1996), with a bootstrap value of 53%, and close to the 86% bootstrap cluster formed by TRYP from *Fusarium oxysporum* (Rypniewski et al. 1993) and SNP1 from *Phaeosphaeria nodorum* (Carlile et al. 2000).

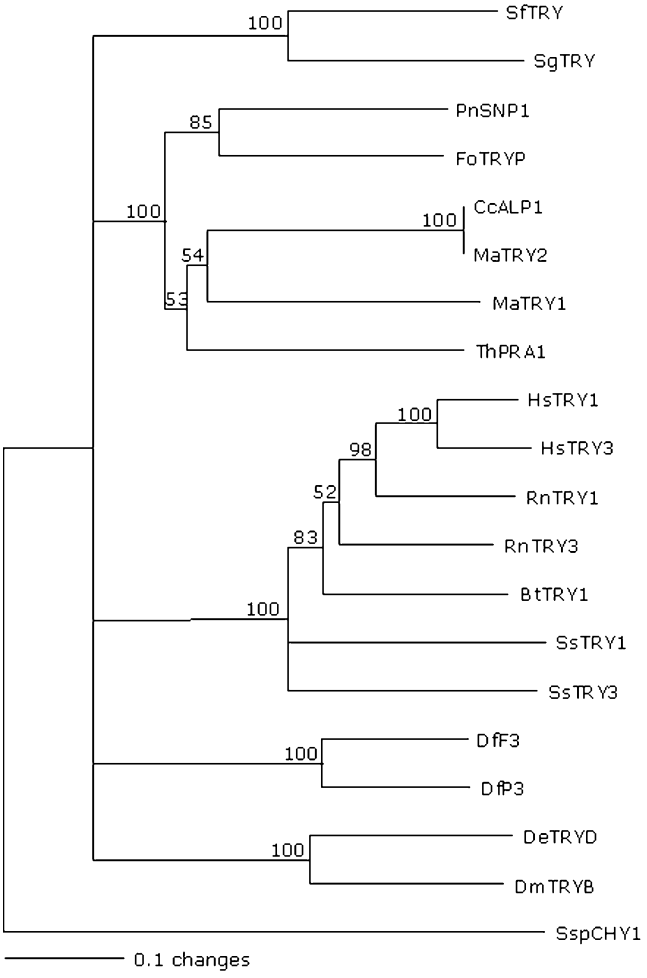

Fig. 3 Phylogenetic relation among PRA1 (accession number: AJ249721) and other trypsins from: *Streptomyces fradiae* (name: TRY; accession number: D16687), *S. glaucescens* (TRY; U13770), *Phaeosphaeria nodorum* (SNP1; AF092435), *F. oxysporum* (TRYP_FUSOX; S36827), *Cochliobolus carbonum* (ALP1; U39500), *Metarhizium anisopliae* (TRY2; AF130865), *M. anisopliae* (TRY1; AJ242736), *Homo sapiens* (TRY1; M22612), *H. sapiens* (TRY3; X15505), *Rattus norvegicus* (TRY1; V01273), *R. norvegicus* (TRY3; M16624), *Bos taurus* (TRY; D38507), *Salmo salar* (TRY1; X70075), *S. salar* (TRY3; X70074), *Dermatophagoides farinae* (DERF3; D63858) and *D. farinae* (DERP3; U11719), *Drosophila erecta* (TRYD; U40653), and *D. melanogaster* (TRYB; M96372). *S. glaucescens* CHY1 (accession number: X74102) was used as outgroup. The phylogenetic tree was obtained by the neighbor-joining method using the Kimura-2-parameter distance. The numbers above the branches indicate the percentages with which a given branch was supported in 500 bootstrap replications

Expression pattern of *pra*1

Samples of *T. harzianum* mycelium grown at different times were collected for Northern analyses. No *pra*1 transcript was detected in mycelium cultivated with glucose (2%) and ammonium (5 g/l) as carbon and nitrogen sources, respectively, at any of the times considered (Fig. 4a, lanes 1–3) either in the presence or absence of fungal cell walls. However, a strong *pra*1 signal was observed under carbon or nitrogen starvation at 4 h (after this time the transcript level decreased) (Fig. 4a, lanes 4–6 and 13–15, respectively). These data suggest that transcription of *pra*1 is repressed by glucose and inorganic nitrogen. It was of interest to define whether the induction occurs in the presence of fungal cell walls (from *C. acutatum*) or chitin (*N*-acetylglucosamine polymer, Sigma) as carbon sources. As can be observed in Fig. 4a (lanes 7–9), in the presence of cell walls *pra*1 mRNA reached the highest levels detected at 4 h (with a strong decay after 9 h), indicating that cell walls or a derived compound induce *pra*1 expression and that mRNA accumulation is not simply due to the lack of glucose as carbon source. From mycelium cultivated with chitin, *pra*1 transcript levels were similar to those obtained with fungal cell walls (lanes 10–12).

The effect of ambient pH on *pra*1 expression was also considered (Fig. 4b). Induction by cell walls or chitin (lanes 3 and 4, respectively) was completely turned off in media buffered at pH 2.5 (lanes 8 and 9) (with no pH control the media had generally a final pH around 5.0, except for cultures with glucose and ammonium, in which the pH was close to 3.5). Furthermore, when mycelium was grown in the absence of glucose (lane 2), the effect of carbon derepression could be completely switched off by changing the pH of the medium to acidic (lane 7). Similarly, *pra*1 transcripts could not be detected in nitrogen-derepressed mycelium under acidic conditions (lane 10). These results suggest a pH regulation for *pra*1 that overrides induction by cell walls or chitin and derepression by carbon or nitrogen starvation.

Effect of PRA1 on egg hatch of *M. incognita*

The study of *M. incognita* egg hatch was carried out in SDDW and other treatment solutions at 25°C (Fig. 5). Percentage hatch, determined by the AUCPH as well as the final cumulative percentage hatch after 25 days of incubation, was significantly influenced ($P<0.05$) by treatments with PRA1. The maximum AUCPH (831.23) and the maximum egg hatch (49.3% of the initial egg number) were obtained in the control (SDDW), while pure preparations of PRA1 at 75–300 ppm reduced AUCPH and final cumulative percentage hatch of *M. incognita* eggs by 39.4 and 43.2%, respectively, compared to the SDDW control. In contrast, a boiled solution of PRA1 did not influence ($P>0.05$) egg hatch of *M. incognita*. In addition, the effect of this trypsin-like protease was compared with that of a commercial trypsin of mammalian origin (bovine trypsin, Sigma). The mammalian trypsin was applied in the assay at the same level of activity as PRA1 (equivalent to 150 ppm PRA1). In this case, the AUCPH and the final cumulative percentage hatch of *M. incognita* eggs were also reduced significantly ($P<0.05$), by 7.6 and 9.8%, respectively, compared with eggs incubated in SDDW. However, the effect was also significantly lower ($P<0.05$) than that observed with PRA1 (Fig. 5). A significant increase ($P<0.05$), 52.7 and 56.9%, of AUCPH and final cumulative percentage hatch, respectively, was also detected using crude culture filtrate

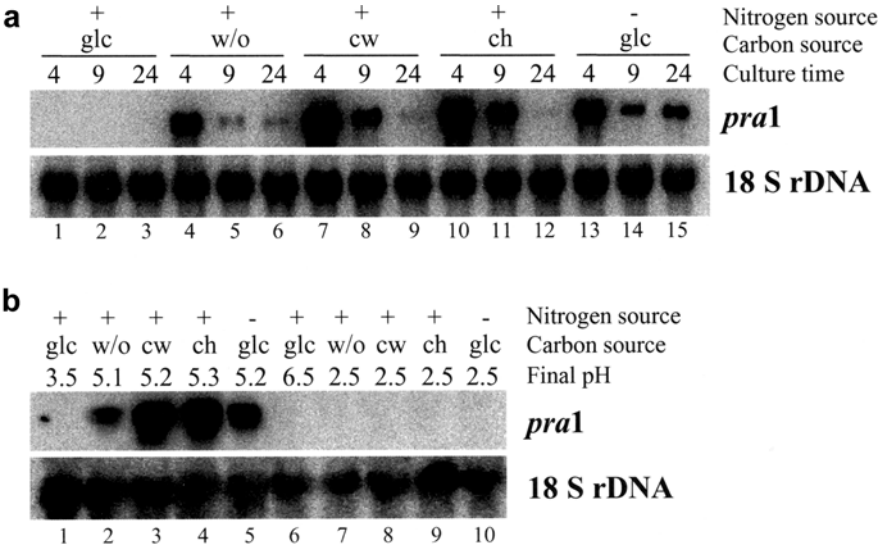

Fig. 4 Northern blot analysis of *pra*1 expression. The experiment was carried out with total RNA (20 µg) extracted from mycelia of *T. harzianum* CECT 2413 grown in MM under the following conditions: 2% glucose (glc); absence of carbon source (w/o); 0.5% fungal cell walls (cw); 1% chitin (ch); standard nitrogen source (ammonium sulfate, 5 g/l) (+); nitrogen starvation (50 mg/ml) (−). **a** Mycelia cultivated for 4–24 h in non-buffered media. **b** Mycelia cultivated for 4 h in non-buffered media (*lanes 1–5*), and buffered media (*lanes 6–10*). The 557-bp PCR product from *pra*1 was used as probe. Radish 18S rDNA was used as loading control

from *T. harzianum* CECT 2413 (500 ppm of total protein), suggesting the presence of other proteins able to complete and/or enhance PRA1 nematicidal effect.

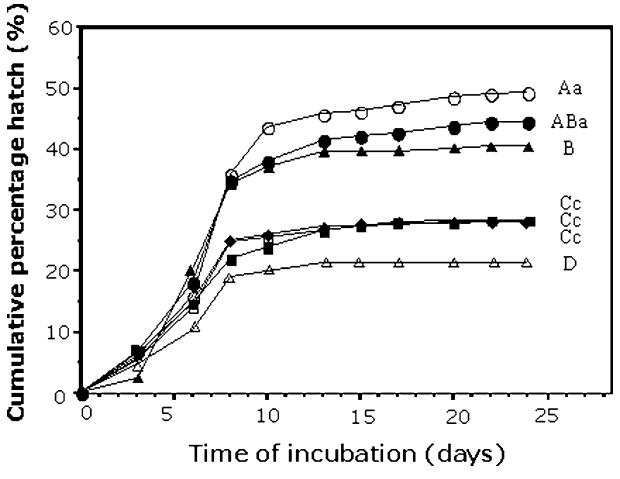

Fig. 5 Effect of PRA1 on egg hatch of *Meloidogyne incognita*. Incubation was carried out at 25°C for 25 days with the following additions: PRA1, containing 75 (*open squares*), 150 (*filled diamonds*), and 300 (*filled squares*) ppm, respectively; 300 ppm of heat-inactivated PRA1 (*filled circles*); trypsine-bovine (Sigma) (*filled triangles*); 500 ppm of crude culture filtrate (*open triangles*); and distilled water (SDDW) (*open circles*). Numbers of J2s that emerged were recorded at 2- to 3-day intervals. Displayed values represent a mean of six replicates. The areas under cumulative percentage hatch (AUCPH) of curves or final hatch curves for each treatment combination followed by the same upper-case or lower-case letters, respectively, do not significantly differ ($P>0.05$), according to Fisher's protected LSD test

Discussion

As suggested previously (Brants et al. 2000; Sharon et al. 2001), the proteolytic and chitinolytic activities produced by *Trichoderma* may be responsible for the biocontrol activity against nematodes described for different strains (Sharon et al. 2001; Spiegel and Chet 1998; Windham et al. 1993). The level of proteolytic activity seems to correlate with nematode control abilities (Ahman et al. 2002), and there is evidence supporting the involvement of subtilisin PRB1 in nematode control by *T. atroviride* IMI 206040 (Sharon et al. 2001). Our results show that egg hatch of *M. incognita* is greatly reduced by both culture filtrates of *T. harzianum* CECT 2413 and pure PRA1 preparations (Fig. 5), indicating that hydrolytic activities produced by this isolate of *T. harzianum* and, specifically PRA1, may offer a promising basis for developing alternatives for the control of root-knot nematodes. Our report on protease PRA1 describes the first gene product from *Trichoderma* spp. showing direct activity by itself against nematodes, and identifies trypsin-like proteases as novel determinants for fungal nematotoxic activities, which have thus far been attributed only to subtilisin proteases (Ahman et al. 2002; Bonants et al. 1995; Segers et al. 1994; Sharon et al. 2001).

Antifungal activities displayed by *Trichoderma* spp. culture filtrates are the result of additive and in some cases synergistic effects of different enzymes (Lorito 1998). The increased activity of crude culture filtrates containing protease PRA1 compared to pure preparations of this protein may reflect a similar situation for the nematotoxic effect. As described here, several proteases were detected in *T. harzianum* CECT 2413 culture filtrates, and at least three endochitinases isolated under similar conditions have been reported (De la Cruz et al. 1992). It is worth

mentioning that although none of the protease activities detected in this study seem to correspond to the PRB1 subtilisin described in *T. atroviride*, it is very likely that this protein was present in the culture filtrates described here, as the *prb*1 transcript has been identified in an EST library constructed from the strain and under the conditions used in this work (unpublished results). A similar proteolytic system, including trypsins and subtilisins, acting complementarily during host cuticle penetration, has been reported for the entomopathogenic fungus *M. anisopliae* (St. Leger et al. 1996).

Our results suggest that the combined action of several proteins displaying proteolytic, and probably chitinolytic, activities present in *T. harzianum* culture filtrates (among them and acting as a key player PRA1) is responsible for degradation of the chitin-protein layer in the nematode eggshell, thus affecting the normal embryogenic development of *M. incognita*. This is in agreement with the hypothesis that the main anti-nematode activity of *Trichoderma* spp. takes place in the soil and not within the roots (Brants et al. 2000). Although our experiments did not show a complete inhibition of egg hatch, the significant reduction detected could be complementary with other control measures, such as chemical nematicides. Thus, the synergistic interaction of enzymatic activity with nematicides might lower the dosage of chemical treatments required for the effective control of nematode populations.

The expression profile of *pra*1 (Fig. 4) is very similar to those of other genes coding for proteins involved in mycoparasitic activities in *Trichoderma* spp. (Donzelli and Harman 2001; Flores et al. 1997; Olmedo-Monfil et al. 2002; Viterbo et al. 2002). This fact and the affinity and activity on fungal cell walls suggest that PRA1 is involved in mycoparasitic processes. Although no significant antifungal activity was detected when pure PRA1 preparations were incubated alone with different phytopathogenic fungi, we do not rule out its antifungal effect when combined with other cell-wall-degrading enzymes from *Trichoderma* spp. A role for *T. atroviride* subtilisin PRB1 protease in mycoparasitic action has been reported (Cortes et al. 1998; Olmedo-Monfil et al. 2002), although no direct antifungal activity was described for the purified protein. Protease activities produced by *Trichoderma* strains during mycoparasitic interactions will probably complement the effect of other lytic enzymes required to make the protein components of fungal cell walls accessible.

The biochemical and sequence characteristics of PRA1 clearly distinguish this protease from PRB1, the other proteolytic enzyme from *Trichoderma* whose sequence has been completed (Geremia et al. 1993). The phylogenetic tree generated with different trypsin-like sequences is in agreement with published data using maximum parsimony analyses of trypsins and chymotrypsins of different origins (Screen and St. Leger 2000). The sequence data for PRA1 and its biochemical properties differentiate this protein from trypsin-like proteases from other sources. The amino acid sequence of PRA1 displays a close relation to those of its fungal counterparts, which are related to insect biocontrol capacities (Smithson et al. 1995; St. Leger et al. 1996). Sequence changes generated by different evolutionary pressures are probably responsible for the differences in antagonistic activity against nematodes, which is significantly higher for PRA1 than for the bovine enzyme (Fig. 5). Strikingly, all trypsin-like enzymes described in fungi are secreted by soil pathogens and/or parasites and presumably play a specific role during infection/parasitic processes (Carlile et al. 2000; Murphy and Walton 1996; Rypniewski et al. 1993; St. Leger et al. 1996).

One of the evolutionary lines followed by an important group of *Trichoderma* haplotypes is the mutualistic association with plants. The colonization of the root system by rhizosphere competent strains of *Trichoderma* results in increased development of root and/or aerial systems and crop yields (Meera et al. 1994). Other activities, such as the induction of plant systemic resistance, have also been described (Yedidia et al. 1999). These facts strongly suggest that during plant-*Trichoderma* interactions, the fungus participates actively in protecting and improving its ecological niche. Antagonism against plant pathogens can also be considered part of this strategy as is also the case for the activity against nematodes of some *Trichoderma* strains. The preservation and improvement of plant growth have an evolutionary advantage as they lead to a better and wider colonizing space for the fungal mutualistic symbiont. In this scenario, the expression and secretion of gene products selected to protect against several threats to the plant (e.g. fungi, nematodes) and/or to increase plant root growth will be favored. Molecular studies of plant-*Trichoderma* interactions may reveal a source of valuable applications in the agro-biotechnology field.

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