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Cerato-platanin, a phytotoxic protein from *Ceratocystis fimbriata*: Expression in *Pichia pastoris*, purification and characterization

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Abstract

Cerato-platanin (CP) is a phytotoxic protein secreted by the Ascomycete *Ceratocystis fimbriata* f.sp. *platani*. This Ascomycete causes canker stain which is a severe disease with a high incidence in the European *Platanus acerifolia*. CP probably plays a role in the disease, eliciting defence-related responses in the host plants. CP is a 120 amino acid protein, containing 40% hydrophobic residues and two S-S bridges. In the EMBL data bank CP is the first member of a new fungal protein family known as the Cerato-Platanin Family. The N-terminal region of CP shows a high similarity with that of cerato-ulmin, a phytotoxic protein produced by the Ophiostoma species and that belongs to the hydrophobin family. Hydrophobins are hydrophobic proteins secreted by many saprophytic or pathogenic fungi and have a remarkable ability to self-assemble into a rodlet structure takes part in physiological and/or pathological processes. The methyltrophic yeast *Pichia pastoris* was used to obtain a high-level expression of recombinant CP (rCP) and the pPIC9 vector was chosen to bring about extra-cellular secretion of the protein. The preliminary structural and functional characterization presented here reveals no significant differences between the native and the recombinant protein. We also show that CP self-assembles in solution. The availability of rCP will allow its three-dimensional structure to be determined, facilitating an understanding of the role of CP in the pathogenesis of canker stain. It is also an excellent model for investigating the mechanism of action of the other proteins related to CP.

Keywords: Hydrophobins; Fungal protein; Canker stain disease; Protein aggregation

Phytopathogenic fungi produce several extra-cellular small proteins having phytotoxic activity. These proteins may induce cell necrosis either by suppressing the plant defensive systems, or by modifying the structure of the plant cellular membrane. In many cases these proteins act as elicitors that stimulate plant defensive systems and inducing the synthesis of molecules such as phytoalexins, that act directly against the pathogen [1]. One of these proteins is cerato-platanin, which is released abundantly in culture by *Ceratocystis fimbriata* f.sp. *platani*, an Ascomycete that causes canker stain in the plane-tree, a severe disease with a high incidence in the European *Platanus acerifolia*. Symptoms of canker stain include foliar wilting and spreading lesions of phloem, cambium and sapwood that may lead to the death of the tree [2]. CP[1] is involved in the development of the disease since it elicits defence-related responses such as phytoalexin synthesis and cell death in both host and non-host plants [3,4].

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[1] *Abbreviations used:* CP, cerato-platanin; rCP, recombinant CP; YNB, yeast nitrogen base; HRP, horseradish peroxidase; RP, reverse-phase; AOX1, alcohol oxidase; MD, minimal dextrose; MM, minimal methanol; Mut^S, methanol utilizing slow; Mut^+, methanol utilizing plus; OD, optical density; PMSF, phenylmethanesulfonyl fluoride; MGY, minimal glycerol yeast; PVDF, polyvinylidene difluoride, TFA, trifluoroacetic acid.

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CP has been purified from the culture filtrate of *C. fimbriata*; it is a 120 amino acid protein (Mw. 12.4 kDa), with 40% hydrophobic residues, two S–S bridges between Cys 20–57 and Cys 60–115, and a 14-residue signal peptide that is removed during secretion of the mature protein [3,5]. In the EMBL data bank, CP is the first member of the Cerato-Platanin Family, a new protein family that consists of seven secreted fungal proteins (EBI-InterPro IPR010829) characterized by a high amino acid sequence similarity, which is unusual among the other, better known, fungal protein families. On the other hand, the proteins in this family are not invariably characterized by a clear functional similarity although, in some cases, they are involved in phyto-pathological phenomena and immunological reactions. For example, the product of the *snodprot1* gene from *Phaeosphaeria nodorum* is expressed when this fungus infects wheat leaves [6]; the allergen Asp f13 causes an allergic reaction in humans [7]; the CS antigen is produced by *Coccidioides immitis*, which is the causative agent of a human respiratory disease [8] and the sp1 protein by *Leptosphaeria maculans*, which is the blackleg pathogen of *Brassica napus* [9].

CP also shares some structural and functional properties in common with the hydrophobins; for example it has a high similarity with the phytotoxic protein cerato-ulmin [10,11]. Hydrophobins are small to medium size (75–120 amino acids) hydrophobic proteins with a low sequence similarity and they are produced by many saprophytic or pathogenic fungi where they play a role in a broad range of growth and development processes [12]. Hydrophobins are usually secreted into the medium and are also localized on the cell walls of hyphae and conidia where they mediate the interaction between the fungus and the environment. On aerial hyphae they form a water-repellent coating characterized by a typical rodlet pattern that reduce the surface tension, allowing the fungus to leave the liquid medium and to grow in the air [13]. Rodlets are also important in the first stage of fungal pathogenesis, when the fungus must adhere to the hydrophobic surface of the plant before it can penetrate and infect the host [19]. Finally, hydrophobins have the remarkable ability *in vitro*, to self-assemble into a polymeric amphipathic monolayer at the hydrophobic/hydrophilic interface that is similar to the rodlet pattern on the surface of aerial hyphae [14,15]. For some highly hydrophobic members of this protein family, self-assembly is characterized by an increase in the β-sheet content and by the formation of amyloid-like structures [16–18].

To explore the role played by CP in canker stain, for which significant amounts of protein are needed, we aimed to produce CP by recombinant methods. *Escherichia coli*, the most common heterologous protein expression system, does not correctly process many polypeptides, so that proteins are formed which are insoluble and incorrectly folded. In the case of the recombinant CP we were able to obtain the correct product only after a refolding step and at low yields [5]. The methylotrophic yeast *Pichia pastoris* is known to be an efficient expression system for proteins containing Cys residues, it can able to produce soluble proteins with a very high yield and having the right post-translational maturation [20]. In the present work we report the expression of rCP in *P. pastoris* and some details of the structure and biological activity of rCP are compared with those of the native CP.

Materials and methods

Materials

The *Pichia* expression kit, restriction enzymes, T4-DNA ligase and *Taq* DNA polymerase were purchased from InVitrogen (San Diego, CA, USA). The host *P. pastoris* strain GS115 (*his4*) from the *Pichia* expression kit was taken for expression of CP and grown, transformed and analyzed according to the manufacturer's instructions [21]. The shuttle vector pPIC9 carrying the secretion signal of the α-mating factor sequence from *S. cerevisiae* was used.

Escherichia coli DH5α (InVitrogen) was used for routine plasmid amplification, the pGEX-2T was from Pharmacia (Uppsala, Sweden) and the pGEM-T Easy vector was from Promega (Madison, WI, USA).

Peptone, tryptone, yeast extract, agar-Y, yeast nitrogen base (YNB) with ammonium sulphate without aminoacid and dextrose , D-biotin and L-histidine were from BIO 101 system inc. (Carlsband, CA, USA); D-glucose, methanol and glycerol were purchased from Sigma-Fluka (St. Louis, MO, USA). The MiniElute Gel Extraction kit, QUIAQuick Gel Extraction kit, HiSpeed Plasmid Midi kit and the PCR Purification kit were purchased from Qiagen (Valencia, CA, USA); the GenElute Plasmid Miniprep kit was from Sigma. The restriction enzymes were from In vitrogen.

PCR amplification was performed with a Gene Amp PCR System 2400 from Perkin Elmer (Wellesley, MA, USA). A 1 Kb plus DNA Ladder (InVitrogen) was used for 1% agarose gel electrophoresis. Protein markers and the Coomassie blue stain reagent (Bio-Rad, Hercules, CA, USA) were used for SDS–PAGE gel electrophoresis; horseradish peroxidase (HRP)-conjugated secondary antibodies and PVDF membranes were from Amersham Biosciences (Piscataway, NJ, USA); the enhanced chemiluminescence (ECL) system for visualizing Western blot was purchased from Pierce (Rockford, IL, USA).

Protein concentration was determined using the Bicinchoninic acid method from Pierce. The semi-preparative Reverse-Phase (RP) HPLC column (C4, 5 μm, 250 × 10 mm) was from Vydac (Grace, Columbia, MD, USA).

The Bio-Gel P-10 column and the Sephadex G75 column employed in the purification of the native protein were from Bio-Rad and Pharmacia, respectively.

The YM-3 membrane and the ultra-filtration unit were from Millipore (Bedford, MA, USA). All reagents were of analytical grade unless otherwise stated.