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Phytomonas serpens: cysteine peptidase inhibitors interfere with growth, ultrastructure and host adhesion

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Abstract

In this study, we report the ultrastructural and growth alterations caused by cysteine peptidase inhibitors on the plant trypanosomatid *Phytomonas serpens*. We showed that the cysteine peptidase inhibitors at 10 μM were able to arrest cellular growth as well as promote alterations in the cell morphology, including the parasites becoming short and round. Additionally, iodoacetamide induced ultrastructural alterations, such as disintegration of cytoplasmic organelles, swelling of the nucleus and kinetoplast–mitochondrion complex, which culminated in parasite death. Leupeptin and antipain induced the appearance of microvillar extensions and blebs on the cytoplasmic membrane, resembling a shedding process. A 40 kDa cysteine peptidase was detected in hydrophobic and hydrophilic phases of *P. serpens* cells after Triton X-114 extraction. Additionally, we have shown through immunoblotting that anti-cruzipain polyclonal antibodies recognised two major polypeptides in *P. serpens*, including a 40 kDa component. Flow cytometry analysis confirmed that this cruzipain-like protein has a location on the cell surface. Ultrastructural immunocytochemical analysis demonstrated the presence of the cruzipain-like protein on the surface and in small membrane fragments released from leupeptin-treated parasites. Furthermore, the involvement of cysteine peptidases of *P. serpens* in the interaction with explanted salivary glands of the phytophagous insect *Oncopeltus fasciatus* was also investigated. When *P. serpens* cells were pre-treated with either cysteine peptidase inhibitors or anti-cruzipain antibody, a significant reduction of the interaction process was observed. Collectively, these results suggest that cysteine peptidases participate in several biological processes in *P. serpens* including cell growth and interaction with the invertebrate vector.

Keywords: *Phytomonas serpens*; Trypanosomatids; Cysteine peptidase inhibitors; Peptidases; Cell surface; Growth; Cellular interaction; Ultrastructural alterations; Cruzipain

1. Introduction

Trypanosomatids are flagellate parasites with a wide range of hosts including insects, mammals, plants and other protists. Plant trypanosomatids are parasites of several families of plants with a wide geographical distribution, being present in South America, Africa, Europe and Asia (Dollet, 1984; Camargo et al., 1990). Infection of plants with trypanosomatids has been known since 1909 (Lafont, 1909). Immediately after their discovery, Donovan proposed the generic name *Phytomonas* to distinguish them from trypanosomes isolated from animals (Donovan, 1909).

The plant flagellates inhabit the phloem (phloemicola), latex (lacticola), flowers (floricola) and fruits (fructicola) of many plant families (Dollet, 1984; Camargo et al., 1990; Camargo, 1999). In many cases, this parasitism exists without any apparent pathogenicity (Cunha et al., 2000). However, these flagellates have attracted attention because they can be responsible for significant economic losses in agriculture. For instance, phloem-dwelling phytomonads cause acute and chronic yellowing of leaves in coffee plants, 'hartrot' in coconut palms and 'marchitez wilt' in oil palms, while the latex-associated *Phytomonas françai* causes 'chochamento das raízes' (empty roots) in manioc (reviewed by Camargo, 1999). These parasites are transmitted by phytophagous hemipteran

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insects of the families Coreidae, Lygaeidae, Pyrrhocoridae and Pentatomidae (Camargo, 1999). In addition, a recent report showed that *Phytomonas serpens*, a tomato parasite, shares antigens with *Trypanosoma cruzi*, which were strongly recognised by human sera from patients with Chagas' disease, and induced a protective immunity in BALB/c mice (Breganó et al., 2003).

The functionally diverse cysteine peptidases of various pathogens have received special attention as potential targets for chemotherapeutic intervention because they play important roles in facilitating the survival and growth of the parasites in hosts (Sajid and McKerrow, 2002). For instance, our research group demonstrated that cysteine peptidases are preferentially expressed in virulent as opposed to avirulent *Leishmania amazonensis* promastigotes (Soares et al., 2003). Additionally, *Leishmania mexicana* null mutants for the multicopy cysteine peptidase gene family *lmpcb* have their infectivity to macrophages reduced by 80% and produced subcutaneous lesions in BALB/c mice at a slower rate than wild-type parasites (Mottram et al., 1996). Proteolytic inhibitor compounds are an interesting alternative to facilitate the cost-effective development of new anti-parasitic chemotherapy (Selzer et al., 1999; Cazzulo et al., 2001). In this sense, cysteine peptidase inhibitors (CPIs) have been shown to kill *T. cruzi* (Ashal et al., 1990; Franke de Cazzulo et al., 1994), *Trypanosoma congolense* (Mbawa et al., 1992), *Trypanosoma brucei* (Troeberg et al., 1999), *Leishmania major* (Selzer et al., 1999) and *Trichomonas vaginalis* (Irvine et al., 1997).

In the present work, the susceptibility of *P. serpens* to a panel of five different CPIs was determined by analyzing their effects on the cellular growth pattern, on the ultrastructural level and on the adhesion index to explanted salivary glands of the phytophagous insect *Oncopeltus fasciatus*. In addition, the cellular proteolytic profile was analysed including the relationship of *P. serpens* proteins to cruzipain, the major cysteine peptidase from *T. cruzi* (reviewed by Cazzulo et al., 2001).

2. Material and methods

2.1. Chemicals

Media constituents, reagents used in electrophoresis, buffer components, nitrocellulose membrane and reagents for chemiluminescence detection were purchased from Amersham Life Science (Little Chalfont, England). Low molecular mass standards were acquired from GIBCO BRL (Grand Island, NY, USA). Dithiothreitol (DTT), gelatin, all proteolytic inhibitors (antipain, cystatin, leupeptin, *trans*-epoxysuccinyl L-leucylamido-(4-guanidino) butane [E-64], iodoacetamide, 1,10-phenanthroline and phenyl-methyl sulfonyl-fluoride [PMSF]) and the secondary antibodies were purchased from Sigma Chemical Co. (St Louis, MO, USA). The anti-cruzipain polyclonal antibody was a gift from Dr Juan-Jose Cazzulo (Instituto de Investigaciones Biotecnologicas, Universidad Nacional de General San Martin, Buenos Aires, Argentina). All other reagents were analytical grade.

2.2. Microorganisms and culture conditions

Phytomonas serpens (isolate 9T; CT-IOC-189), isolated from tomato (*Lycopersicon esculentum*), was provided by Dr Maria Auxiliadora de Sousa (Coleção de Tripanosomatídeos, Instituto Oswaldo Cruz, Rio de Janeiro). The plant flagellate was grown in 50 ml Erlenmeyer flasks containing 20 ml of brain heart infusion (BHI) medium, pH 7.4, at 26 °C (Santos et al., 2002). The flagellate *T. cruzi* (Dm28c strain) is part of our laboratory collection, and epimastigote forms were cultured in liver infusion trypticase (LIT) medium, for 72 h at 26 °C (Souto-Padrón et al., 1990). Both culture media were supplemented with 10% (v/v) heat-inactivated FCS.

2.3. Effects of CPIs on the growth rate

The experiments were performed in 10×100 mm glass tubes containing 1 ml of BHI+FCS medium. The inoculum consisted of 10% (v/v) of a 48 h culture containing about 2.0×10^6 *P. serpens* cells. All CPIs (antipain, cystatin, leupeptin, E-64 and iodoacetamide) were dissolved in water at 1 mM, and then filter-sterilised in 0.22 μm membranes (Millipore). The parasites were grown at 26 °C for periods ranging from 24 to 96 h, in the absence or presence of each peptidase inhibitor at 10 μM. Alternatively, parasites grown for 48 h in the presence of each peptidase inhibitor were washed five times in cold PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.2) prior to resuspension in a drug-free fresh medium and allowed to grow for another 48 h. Cellular growth was estimated daily by counting the flagellates in a Neubauer chamber (Santos et al., 2003).

2.4. Effects of CPIs on the ultrastructure of the flagellates

Control and CPI-treated phytomonads were grown for 48 h at 26 °C, collected by centrifugation for 15 min at 1500×*g* at 4 °C, washed three times with PBS and fixed for 2 h with 1.5% glutaraldehyde and 4% paraformaldehyde in 0.05 M cacodylate buffer pH 7.2 containing 1 mM $CaCl_2$. The cells were then rinsed in cacodylate buffer and postfixed for 1 h in a solution containing 1% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer pH 7.2 supplemented with 1 mM $CaCl_2$. After being rinsed in the same buffer, the parasites were dehydrated in graded acetone and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss Em-900 transmission electron microscope operating at 80 kV (Souto-Padrón et al., 1990).

2.5. Detergent extraction of proteins

Phytomonas serpens cells (1.0×10^8) were harvested as described before and then lysed in 200 μl of 2% (w/v) precondensed Triton X-114 in TBS (150 mM NaCl, 10 mM Tris, pH 7.4) in a vortex by alternating 1 min shaking and 2 min cooling intervals. Insoluble material was precipitated (10,000×*g* for 30 min at 4 °C) and the supernatant was incubated