Recombinant expression and characterization of a *Xylella fastidiosa* cysteine protease differentially expressed in a nonpathogenic strain

Viviane Nogaroto[1], Sandra A. Tagliavini[2], Andréia Gianotti[1], Angela Mikawa[2], Nilana M.T. Barros[3], Luciano Puzer[3], Adriana K. Carmona[3], Paulo I. Costa[4] & Flávio Henrique-Silva[1]

[1]Laboratory of Molecular Biology, Department of Genetics and Evolution, Federal University of São Carlos, São Carlos, SP, Brazil; [2]Institute of Chemistry, UNESP, Araraquara, SP, Brazil; [3]Department of Biophysics, Unifesp, São Paulo, SP, Brazil; and [4]Department of Clinical and Toxicological Analyses of the Faculty of Pharmaceutical Sciences, São Paulo State University; São Paulo, SP, Brazil

Correspondence: Flávio Henrique-Silva, Laboratório de Biologia Molecular-DGE, Universidade Federal de São Carlos, Rod: Washington Luis, Km 235, CEP: 13565-905, São Carlos, SP, Brazil. Tel.: +55 16 3351 8378; fax: +55 16 3351 8377; e-mail: dfhs@power.ufscar.br

Received 25 January 2006; revised 16 May 2006; accepted 31 May 2006.
First published online 5 July 2006.

DOI:10.1111/j.1574-6968.2006.00348.x

Editor: Reggie Lo

Keywords
Xylella fastidiosa; citrus variegated chlorosis; phytopathogen; recombinant expression; cysteine protease; xylellain.

Abstract

Xylella fastidiosa is a xylem-limited, Gram-negative bacterium responsible for citrus variegated chlorosis (CVC) in sweet oranges. In the present study, we present the recombinant expression, purification and characterization of an *X. fastidiosa* cysteine protease (dubbed Xylellain). The recombinant Xylellain ($_{HIS}$Xylellain) was able to hydrolyze carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin (Z-FR-MCA) and carbobenzoxy-Arg-Arg-7-amido-4-methylcoumarin (Z-RR-MCA) with similar catalytic efficiencies, suggesting that this enzyme presents substrate specificity requirements similar to cathepsin B. The immunization of mice with $_{HIS}$*Xylellain* provided us with antibodies, which recognized a protein of *c*. 31 kDa in the *X. fastidiosa* pathogenic strains 9a5c, and *X. fastidiosa* isolated from coffee plants. However, these antibodies recognized no protein in the nonpathogenic *X. fastidiosa* J1a12, suggesting the absence or low expression of this protein in the strain. These findings enabled us to identify Xylellain as a putative target for combating CVC and other diseases caused by *X. fastidiosa* strains.

Introduction

Xylella fastidiosa is a xylem-limited, Gram-negative bacterium responsible for a large number of economically important plant diseases, such as Pierce's disease in grapevines (Mollenhauer & Hopkins, 1974; Davis *et al*., 1978), citrus variegated chlorosis (CVC) in sweet oranges (Rossetti *et al*., 1990; Chang *et al*., 1993) and leaf scorch diseases in other plants, including almond, plum, oleander, mulberry and coffee (Purcell & Hopkins, 1996). In all cases, *X. fastidiosa* infects the plant xylem and impairs fruit production.

CVC is considered to be potentially the most devastating citrus disease. Symptoms were first observed in the states of Sao Paulo and Minas Gerais, Brazil in 1987, and the identification of *X. fastidiosa* as the causative pathogen of CVC occurred 6 years later in 1993 (Chang *et al*., 1993; Hartung *et al*., 1994). The disease is responsible for production losses mainly in Sao Paulo and has been identified in more than 90% of the orchards in the state (Catani *et al*., 2004). CVC causes annual losses of about $100 million to the citrus industry in Brazil alone (de Souza *et al*., 2003).

The complete genome sequence of the *X. fastidiosa* strain 9a5c was published in 2000 and represents the first complete genome sequence of a phytopathogen (Simpson *et al*., 2000). Recently, the genome sequence of the *X. fastidiosa* Temecula strain isolated from a naturally infected grapevine with Pierce's disease was published (Van Sluys *et al*., 2003). The *X. fastidiosa* 9a5c and Temecula strains have identical metabolic functions and are likely to use a common set of genes in plant colonization and pathogenesis, permitting the convergence of functional genomic strategies (Van Sluys *et al*., 2003). Several *X. fastidiosa* strains have had their genomes completely or partially sequenced. However, little information on the nonpathogenic *Xylella* strain genome composition is available. Such information would contribute toward more direct insights on pathogenicity mechanisms (Koide *et al*., 2004).

Despite knowledge on the *X. fastidiosa* strain 9a5c genome, studies on the biochemical characterization of proteins involved in the pathogenicity of this organism are still scarce. A proteome analysis was recently performed to

evaluate the proteins expressed by this pathogen (Smolka *et al.*, 2003). Particular attention has been paid to adhesion and secreted proteins because of their possible role in bacterial pathogenesis and their usefulness as molecular targets for understanding and controlling the disease (Smolka *et al.*, 2003).

Proteins such as proteases, cellulases and lipases may be involved in the infection process by disrupting plant tissue and allowing the spread of bacteria throughout the vascular system (Hopkins, 1989). Proteases have been divided into five main groups on the basis of the catalytic mechanism used during the hydrolytic process: serine, aspartyl, metallo, threonine and cysteine proteases (Barrett *et al.*, 2004). The interest in cysteine proteases as chemotherapeutic targets stems from the recognition that they are critical to the life cycle or pathogenicity of a large number of parasites (Sajid & McKerrow, 2002). Cysteine proteases from parasites play key roles in immunoevasion, enzyme activation, virulence, tissue and cellular invasion, as well as excystment, hatching and molting (Lecaille *et al.*, 2002; Sajid & McKerrow, 2002).

In the present study, we present the recombinant expression, purification and characterization of an *X. fastidiosa* cysteine protease (dubbed Xylellain). This protein may be involved in *X. fastidiosa* pathogenicity and therefore constitutes a probable target for combating CVC and other diseases.

Materials and methods

Bacterial strains and growth conditions

Strains of *X. fastidiosa* isolated from symptomatic coffee plants (*Coffea arabica* L.), triply cloned *X. fastidiosa* strains 9a5c (Li *et al.*, 1999) and J1a12 (Teixeira *et al.*, 2004) isolated from sweet oranges (*Citrus sinensis* L.) were grown in a periwinkle wilt broth medium (Davis *et al.*, 1981) at 28 °C. The strain J1a12 (nonpathogenic) causes few or no CVC symptoms when inoculated in citrus and tobacco plants, despite its isolation from CVC symptomatic *C. sinensis* (L.) (Koide *et al.*, 2004; Teixeira *et al.*, 2004).

ORF amplification and construction of the expression vector

The 816 bp coding region for the cysteine protease was amplified by PCR using the following primers (CPXYLF: 5′ cat atg caa act gtc ttg aaa ag 3′; CPXYLR: 5′ gga tcc tta ttt tct gac ggt ctt a 3′) and DNA was extracted from the *X. fastidiosa* 9a5c strain. The primers were designed based on the sequence deposited in the GenBank (GenBank accession No. AE003869, locus_tag XF_0156, position 159327–160142, complement). Twenty nanogram template DNA, 1 U *Taq* DNA Polymerase (Fermentas), 1 × reaction buffer containing 100 mM Tris-HCl, pH 8.5, and 500 mM KCl, 0.2 mM dNTPs and 3 mM $MgCl_2$ were used in the PCR reaction. Amplification was performed in a PTC-100 TM MJ research thermocycler programmed for (1 ×) 94 °C 5 min, (35 ×) 94 °C 1 min, 55 °C 1 min, 72 °C 1 min, and (1 ×) 72 °C 5 min. The *Nde*I and *Bam*HI sites were included in the primers for cleavage and insertion in the expression vector pET28a, which was also digested with the same enzymes.

The insert was then subcloned in pET28a in a frame with an N-terminal poly-His coding sequence, generating pET28xylCP. Recombinant plasmids were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1997), using the ET-Dyenamic terminator in an ABI Prism 377 DNA sequencer.

Expression of His-tagged Xylellain ($_{HIS}$Xylellain)

The pET28xylCP vector was used to transform chemically competent *Escherichia coli* BL21 (DE3) cells. Transformed cells were grown at 37 °C and 200 r.p.m. in a selective medium containing kanamycin (25 μg μL^{-1}) until $OD_{600\,nm}$ was 0.5. Expression of the $_{HIS}$*Xylellain* was induced at 30 °C by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Aliquots of the induced culture were taken 1, 2, 3, 4 and 16 h after IPTG addition and analyzed in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The induced cells were harvested by centrifugation (8000 ***g***, 4 °C for 5 min), resuspended in Tris-8.0 buffer (10 mM Tris-HCl, 100 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and disrupted by sonication, using ten 1 min pulses. The lysate was centrifuged at 13 000 ***g***, 4 °C for 15 min, and the supernatant and precipitate were analyzed in 15% SDS-PAGE (Laemmli, 1970).

Purification of the $_{HIS}$*Xylellain*

The $_{HIS}$*Xylellain* was purified from the supernatant using an affinity nickel resin column Ni-NTA superflow (Qiagen) equilibrated with Tris-8.0 buffer. The column was washed with 25 mL Tris-8.0 buffer (five column volumes) and the protein was eluted with the same buffer containing increased concentrations of imidazole (10, 25, 50, 75, 100, 250 and 500 mM). The purified product was analyzed in 15% SDS-PAGE. The fractions containing the purified protein were dialyzed using MW 14 000 membranes (Spectrum Laboratories). The protein concentration was determined by Bradford's method (Bradford, 1976).

Assays of $_{HIS}$*Xylellain* catalytic activity

The Z-FR-MCA and Z-RR-MCA (MCA,methyl-7-amino-coumarin amide) substrates and the inhibitor E64 ([trans-epoxy-succinyl-L-leucylamido-(4-guanidino)butane]) were purchased from Sigma. The peptide Z-LR-MCA was obtained from Novabiochem. The fluorogenic substrates were assayed in a Hitachi F-2500 spectrofluorometer at 37 °C.

Assays were performed in 50 mM sodium acetate buffer, pH 5.0. The $_{HIS}$*Xylellain* was preactivated in the presence of 2.5 mM DTE for 5 min at 37 °C before the addition of the substrates. Fluorescence changes were continuously monitored at $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm. The molar concentration of the $_{HIS}$*Xylellain* was determined by active site titration with E64, according to Barrett & Kirschke (1981). The apparent second-order rate constant k_{cat}/K_m was determined under pseudo-first-order conditions, where $[S] \ll K_m$, performed at two different substrate concentrations and calculated by a nonlinear regression using the *Grafit* program (Leatherbarrow, 1992). In all determinations, errors were less than 5%. Fractions eluted at 100 and 250 mM imidazole from the supernatant of a pET28a-transformed *E. coli* BL21 subjected to separation on an affinity nickel resin column were included in the enzyme assays as a negative control.

pH activity profile

pH dependence was studied at 37 °C by the fluorimetric assay described above, using Z-FR-MCA as substrate. We used a four-component buffer containing 25 mM glycine, 25 mM acetic acid, 25 mM Mes and Tris 75 mM ($3.0 < pH < 7.0$). The enzyme was preactivated with 2.5 mM DTE for 5 min at 37 °C before the addition of the substrate. For each pH, the apparent second-order rate constant (k_{cat}/K_m) was determined at low substrate concentrations, as described above.

Influence of salt on catalytic activity and enzyme inhibition with E64

The influence of NaCl on the catalytic activity of $_{HIS}$Xylellain was investigated over a salt concentration range from 0 to 500 mM. Assays were performed using Z-FR-MCA as substrate at 37 °C in 50 mM sodium acetate, pH 5.0. The enzyme was preactivated in the presence of 2.5 mM DTE for 5 min at 37 °C before the addition of the substrate. Enzymatic activity was followed as described above. k_{cat}/K_m values were calculated by a nonlinear regression using the *Grafit* program (Leatherbarrow, 1992).

$_{HIS}$Xylellain activity in the presence of the specific inhibitor for cysteine proteases E64 was assayed with 10 μM of Z-FR-MCA in 50 mM sodium acetate, pH 5.0, at 37 °C. The enzyme was first preactivated with 2.5 mM DTE for 5 min at 37 °C and then preincubated with 1 μM of E64 for 15 min before the addition of the substrate. The residual activity was followed as described above.

Western immunoblot

Anti-$_{HIS}$Xylellain polyclonal antibodies were raised in mice following a standard procedure (Sambrook & Russel, 1989). The immunization schedule used 50 μg of the purified $_{HIS}$Xylellain along with Freund's complete adjuvant (Sigma). A second injection was performed 6 weeks later using 50 μg of the purified $_{HIS}$Xylellain along with Freund's incomplete adjuvant (Sigma). Ten days later, the immunized animals were bled and the serum was collected through centrifugation at 15 700 ***g*** for 5 min and kept at 4 °C.

For Western immunoblotting, proteins were subjected to electrophoresis in 15% SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting in a buffer containing 200 mM Tris, 50 mM glycine and 20% methanol. The membrane was incubated overnight in a blocking buffer and then washed with PBS (100 mM Tris, 70 mM NaCl, pH 8.0), incubated with anti-$_{HISXylellain}$ antibody (1 : 10 000) for 90 min, and washed as described above. The membrane was then incubated with antimouse IgG (Sigma) for 90 min, washed with phosphate-buffered saline (PBS) and revealed with AP Color Development reagent (Bio Rad).

Results and discussion

Similarity of *X. fastidiosa* cysteine protease to other cysteine proteases

The protein studied in the present study, which we have dubbed Xylellain, is similar to several cysteine proteases from different organisms (Fig. 1). This *X. fastidiosa* cysteine protease showed high similarity with other phytopathogen proteases, such as *X. fastidiosa* Temecula strain, the agent responsible for Pierce's disease in grapevines (Van Sluys *et al.*, 2003) and *Pseudomonas syringae*, which causes bacterial specks in tomatoes (Chen *et al.*, 2000). These alignments showed highly conserved regions within these proteins, including a cysteine-containing motif (GSC) close to the 60th amino acid, suggesting that this cysteine is probably from the catalytic site.

Cloning, expression and purification of the His-tagged Xylellain

The complete ORF sequence was confirmed through nucleotide sequencing and corresponded to the sequence previously annotated as a cysteine protease (Simpson *et al.*, 2000).

The recombinant Xylellain was efficiently expressed in *E. coli* as a His-tagged fusion with a molecular mass of *c.* 33 kDa (corresponding to fusion with the His-tag) (Fig. 2). Most of the recombinant protein was located in the supernatant, specifically in its soluble form. This was achieved by growing the bacteria at 30 °C rather than at the typical temperature of 37 °C, as the latter condition renders a larger amount of protein in inclusion bodies (data not shown). The His-tagged Xylellain was purified through affinity chromatography in a nickel column. The expressed

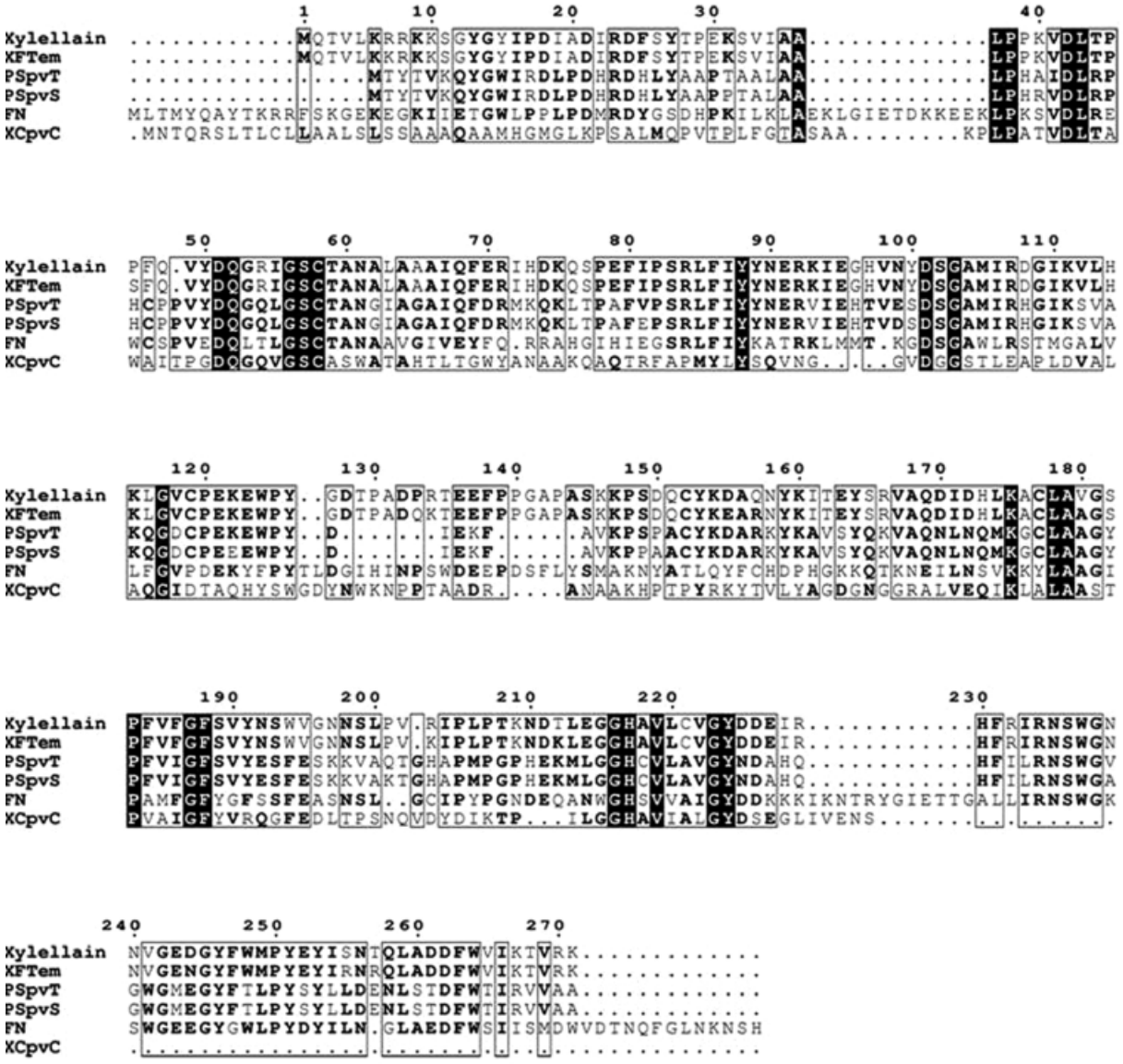

Fig. 1. Alignment between the cysteine protease from *Xylella fastidiosa* strain 9a5c (Xylellain), a similar enzyme from *X. fastidiosa* Temecula (XFTem) and cysteine proteases from other bacteria, such as *Pseudomonas syringae* pv. *tomato* (PSpvT), *Pseudomonas syringae* pv. *syringae* (PSpvS), *Fusobacterium nucleatum* (FN) and *Xanthomonas campestris* pv. *campestris*, showing conserved regions among these proteins. Black boxes indicate identity of above 90% within the sequences, and empty boxes indicate identity between 50% and 90%.

$_{HIS}$Xylellain, specifically linked to the nickel resin through fusion with a histidine tail (~1.5 kDa) originating from pET28a, was eluted from the column with a buffer containing 100, 250 and 500 mM imidazole (Fig. 2).

The purification system was quite efficient, as SDS-PAGE analyses revealed the presence of a single band of $_{HIS}$Xylellain in the collected fractions. The total yield was around 15 mg L^{-1} of culture, used in activity tests and in the structural studies underway.

Activity assays for $_{HIS}$*Xylellain*

The enzymatic activity of $_{HIS}$Xylellain was studied using fluorescent peptides with general structure Z-Xaa-Arg-MCA (Z = carbobenzoxy; Xaa = Arg, Phe or Leu; MCA = 7-amino-4-methylcoumarin) as substrates. Table 1 shows the catalytic efficiencies for the hydrolyses of these peptides by $_{HIS}$Xylellain. The enzyme was activated by sulfhydryl compounds and inhibited by E-64 (Hanada *et al*., 1978), which is an

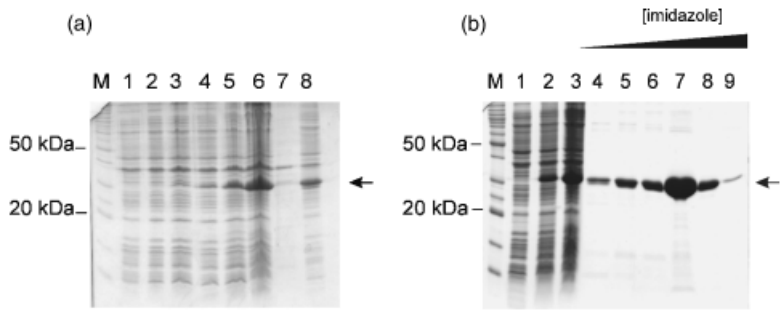

Fig. 2. Analysis of the expression, solubility test and purification of the His-tagged Xylellain in *Escherichia coli*. (a) SDS-PAGE 15% stained with Coomassie blue, showing the induction of $_{HIS}$Xylellain expression with 0.4 mM IPTG. M, molecular mass marker; 1, control without induction; 2, BL21 (DE3) pET28xylCP transformed at 1 h of induction; 3, BL21 (DE3) pET28xylCP transformed at 2 h of induction; 4, BL21 (DE3) pET28xylCP transformed at 3 h of induction; 5, BL21 (DE3) pET28xylCP transformed at 4 h of induction; 6, BL21 (DE3) pET28xylCP transformed at 16 h of induction; 7, pellet; 8, supernatant. (b) SDS-PAGE 15% stained with Coomassie blue, showing induction and subsequent purification of $_{HIS}$Xylellain by affinity chromatography in nickel resin. M, molecular mass marker. 1, control without induction; 2, BL21 (DE3) pET28xylCP transformed at 4 h of induction; 3, BL21 (DE3) pET28xylCP transformed at 16 h of induction; 4 and 5, eluted (100 mM imidazole); 6 and 7, eluted (250 mM imidazole); 8 and 9, eluted (500 mM imidazole).

Table 1. Second-order rate constants (k_{cat}/K_m) for the hydrolysis of fluorogenic substrates by $_{HIS}$Xylellain

Substrate	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Z-FR-MCA	2.3
Z-LR-MCA	1.6
Z-RR-MCA	2.6

Z-FR-MCA, carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin; Z-RR-MCA, carbobenzoxy-Arg-Arg-7-amido-4-methylcoumarin.

effective cysteine protease inhibitor (data not shown). These results, together with the alignment with other cysteine proteases, confirmed that this protease is a cysteine protease, which led us to dub it 'Xylellain'.

According to the Schechter and Berger nomenclature (Schechter & Berger, 1967), the S_2 subsite of cysteine proteases from the papain family is described as the substrate specificity determining binding site (Turk *et al*., 1998), and the majority of the enzymes in this class prefer hydrophobic residues at the P_2 position of the substrates. However, cathepsin B presents the distinguishing feature of hydrolyzing peptides containing either hydrophobic or basic P_2 position residues (Barrett & Kirschke, 1981). The ability of $_{HIS}$Xylellain to hydrolyze Z-FR-MCA and Z-RR-MCA with similar catalytic efficiencies is an indication that this enzyme presents substrate specificity requirements similar to cathepsin B. A more detailed study will be necessary in order to classify Xylellain as a cathepsin B-like enzyme.

The effect of pH and the influence of NaCl on $_{HIS}$Xylellain enzymatic activity were examined using Z-FR-MCA as substrate. $_{HIS}$Xylellain presents better activity at pH 5.0 (Fig. 3). This is characteristic of cysteine proteases, which are generally active in pH ranging from 4.5 to 6.0, and are practically inactive in pH 7.0 (Turk *et al*., 2000). Furthermore, a significant influence of NaCl concentration on the catalytic efficiency of Xylellain was detected, as shown in the inset of Fig. 3.

Expression analysis by Western immunoblot

The immunization of mice with $_{HIS}$Xylellain provided us with antibodies that recognized a protein of approximately 31 kDa in the *X. fastidiosa* pathogenic strain 9a5c and *X. fastidiosa* isolated from coffee plants, confirming the presence of this protein in both strains (Fig. 4). In fact, the antibodies also recognized an *X. fastidiosa* strain isolated from plum plants, and an *X. fastidiosa* strain isolated from grapevines (data not shown). Two bands are visible in the blot, probably due to protein degradation or posttranslational processing. However, this result needs to be investigated in future studies. Interestingly, these antibodies were not able to recognize any protein in the nonpathogenic *X. fastidiosa* J1a12, suggesting the absence or low expression of Xylellain in this strain. This fact may be explained through differences in the promoter or factors involved in the gene transcription, diminishing or abolishing the Xylellain transcription in this nonpathogenic strain. This could also be due to a significant difference between the nonpathogenic xylellain gene and the pathogenic gene, impairing the recognition of protein by the antibody produced using the protein derived from *X. fastidiosa* 9a5c. However, we were able to amplify the complete xylellain gene using DNA from the *X. fastidiosa* J1a12, and after sequencing we confirmed that the ORF is identical to the *X. fastidiosa* 9a5c. On the other hand, the promoter region of J1a12 xylellain gene was also sequenced and presented some differences when compared with 9a5c (data not shown). This result suggests that the difference may be in the promoter region or in some

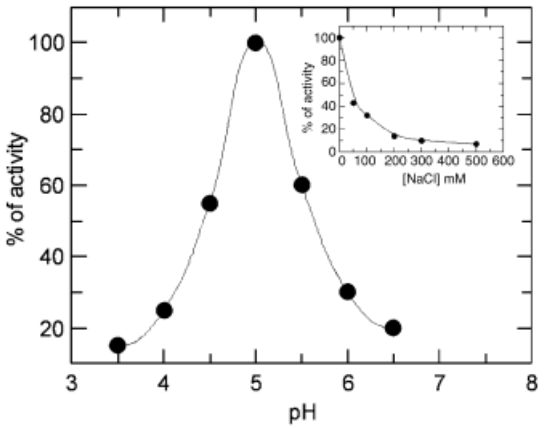

Fig. 3. pH activity profile and influence of NaCl (inset) from Z-FR-MCA hydrolysis by $_{HIS}$Xylellain. The assay conditions are described in the Materials and methods section.

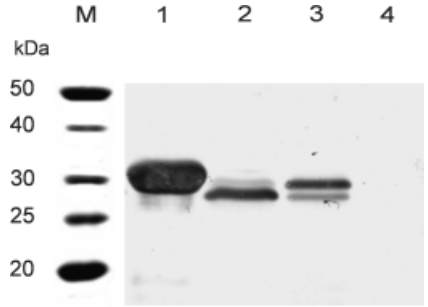

Fig. 4. Immunodetection of recombinant Xylellain and cysteine proteases in protein extracts from different *Xylella fastidiosa* strains. M, molecular mass marker (Invitrogen); 1, $_{HIS}$Xylellain; 2, *X. fastidiosa* strain 9a5c; 3, *X. fastidiosa* isolated from coffee plants; 4, nonpathogenic *X. fastidiosa* strain J1a12.

factor necessary for the gene expression, or even due to a posttranslational modification in Xylellain from the nonpathogenic strain. Future studies should be performed to clarify this point.

In conclusion, the present study has demonstrated the presence of a differentially expressed protein in *X. fastidiosa* pathogenic strains, in comparison to a nonpathogenic strain. This protein was recombinantly expressed in *E. coli* and characterized as a cysteine protease. These findings enabled us to elect this protein, dubbed Xylellain, as a putative target for combating CVC and other diseases caused by *X. fastidiosa* strains.

Acknowledgements

We thank the Brazilian research funding institutions Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, CEPID-CBME Process # 98/14138-2) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Process 470905/2005-2) for financial support. V. Nogaroto is a fellowship recipient of Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We also thank FUNDECITRUS (Fundo de Defesa da Citricultura, Araraquara, SP, Brazil) for providing us with *Xylella fastidiosa* strains.

Authors contribution

V.N. and S.A.T. contributed equally to this study.

References

Barrett AJ & Kirschke H (1981) Cathepsin B, cathepsin H and cathepsin L. *Methods Enzymol* **80**: 535–561.

Barrett AJ, Rawlings ND & Woessner JF (eds) (2004) *Handbook of Proteolytic Enzymes*, 2nd edn, p. 3. Academic Press, London.

Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* **72**: 248–254.

Catani CF, Azzoni AR, Paula DP, Tada SFS, Rosselli LK, de Souza AP & Yano T (2004) Cloning, expression, and purification of the virulence-associated protein D from *Xylella fastidiosa*. *Protein Expr Purif* **37**: 320–326.

Chang CJ, Garnier M, Zreiki L, Rossetti V & Bové JM (1993) Culture and serological detection of the bacterium causing CVC – a strain of *Xylella fastidious*. *Curr Microbiol* **27**: 137–142.

Chen Z, Kloek AP, Boch J, Katagiri F & Kunkel BN (2000) The *Pseudomonas syringae* avrRpt2 gene product promotes pathogen virulence from inside plant cells. *Mol Plant-Microbe Interact* **13**: 1312–1321.

Davis MJ, Purcell AH & Thomson SV (1978) Pierce's disease of grapevines: isolation of the causal bacterium. *Science* **199**: 75–77.

Davis MJ, French WJ & Schaad NW (1981) Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Curr Microbiol* **6**: 309–314.

de Souza AA, Takita MA, Coleta-Filho HD, Caldana C, Goldman GH, Yanai GM, Muto NH, de Oliveira RC, Nunes LR & Machado MA (2003) Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity. *Mol Plant Microbe Interact* **16**: 867–875.

Hanada K, Tamai M, Yamagishi M, Ohmura S, Sawada J & Tanaka I (1978) Isolation and characterization of E-64, a new thiol protease inhibitor. *Agric Biol Chem* **42**: 523–528.

Hartung JS, Beretta MJG, Brlansky RH, Spisso J & Lee RF (1994) Citrus variegated chlorosis bacterium: axenic culture, pathogenicity and serological relationships with other strains of *Xylella fastidiosa*. *Phytopathology* **84**: 591–597.

Hopkins DL (1989) *Xylella fastidiosa*: xylem-limited bacterial pathogen of plants. *Annu Rev Phytopathol* **27**: 271–290.

Koide T, Zaini PA, Moreira LM, *et al.* (2004) DNA microarray-based genome comparison of a pathogenic and a non-pathogenic strains of *Xylella fastidiosa* delineates genes important for bacterial virulence. *J Bacteriol* **186**: 5442–5449.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Leatherbarrow RJ (1992) *Grafit, Version 5.0*, Erithacus software Ltd, Stains, UK.

Lecaille F, Kaleta J & Bromme D (2002) Human and parasitic papain-like cysteine proteases: their role in physiology and pathology and recent developments in inhibitor design. *Chem Rev* **102**: 4459–4488.

Li WB, Zreik L, Fernandes NG, Miranda VS, Teixeira DC, Ayres AJ, Garnier M & Bove JM (1999) A triply cloned strain of *Xylella fastidiosa* multiplies and induces symptoms of citrus variegated chlorosis in sweet orange. *Curr Microbiol* **39**: 106–108.

Mollenhauer HH & Hopkins DL (1974) Ultrastrutural study of Pierce's disease bacterium in grape xylem tissue. *J Bacteriol* **119**: 612–618.

Purcell AH & Hopkins DL (1996) Fastidious xylem-limited bacterial plant pathogens. *Annu Rev Phytopathol* **34**: 131–151.

Rossetti V, Garnier M, Bové JM, Beretta MJD & Teixeira AR (1990) Presence de bacteries dans le xylem d'orangers atteint de chlorose variegee: une nouvelle maladie des agrumes au Brazil. *C R Acad Sci* **310**: 345–349.

Sajid M & McKerrow JH (2002) Cysteine proteases of parasitic organisms. *Mol Biochem Parasitol* **120**: 1–21.

Sambrook J & Russel DW (1989) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York.

Sanger F, Nicklen S & Coulson AR (1997) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci* **74**: 5463–5467.

Schechter I & Berger A (1967) On the size of the active site in proteases. I Papain. *Biochem Biophys Res Commun* **27**: 157–162.

Simpson AJG, Reinach FC, Arruda P, *et al.* (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* **406**: 151–157.

Smolka MB, Martins D, Winck FV, *et al.* (2003) Proteome analysis of the plant pathogen *Xylella fastidiosa* reveals major cellular and extracellular proteins and a peculiar codon bias distribution. *Proteomics* **3**: 224–237.

Teixeira DC, Rocha SRP, Santos MA, Mariano AG, Li WB & Monteiro PB (2004) A suitable *Xylella fastidiosa* CVC strain for post-genome studies. *Curr Microbiol* **49**: 396–399.

Turk D, Guncar G, Podobnik M & Turk B (1998) Revised definition of substrate binding sites of papain-like cysteine proteases. *Biol Chem* **379**: 137–147.

Turk B, Turk D & Turk V (2000) Review: lysosomal cysteine proteases: more than scavengers. *Biochem Biophys Acta* **1477**: 98–111.

Van Sluys MA, de Oliveira MC, Monteiro-Vitorello CB, *et al.* (2003) Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *J Bacteriol* **185**: 1018–1102.