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Characterization of an extracellular serine protease of *Fusarium eumartii* and its action on pathogenesis related proteins

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Abstract

Proteases have been proposed as part of the invasion strategies of some pathogenic fungi. In this work, a serine protease produced by the phytopathogenic fungus *Fusarium solani* f. sp. *eumartii* was purified and characterized. Purification of the enzyme was accomplished by gel filtration through a Superose 12 column, followed by hydrophobic interaction chromatography in Phenyl Superose and gel filtration chromatography through Superdex 75. Analysis of the purified enzyme by SDS/PAGE without heat treatment, revealed a single band, which corresponded to the proteolytic activity detected by zymogram. When this protein was subjected to denaturing conditions, two major polypeptides of approximately 30 and 33 kDa were revealed. The N-terminal amino acid sequence of one of these polypeptides showed a high similarity with fungal mature serine proteases of the subtilisin family. This protease hydrolysed *in vitro*, specific polypeptides of potato intercellular washing fluids and cell walls. The protease was also able to degrade pathogenesis-related proteins from the intercellular washing fluids. The role of this serine protease as part of the fungal strategy to colonize potato tuber tissues is discussed.

Abbreviations: DFP – diisopropylfluorophosphate; E-64 – L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane; FESP – *Fusarium* extracellular serine protease; IWF – intercellular washing fluid; PR proteins – pathogenesis-related proteins; PMSF – phenylmethylsulfonyl fluoride.

Introduction

Fusarium solani (Mart.) Sacc. f. sp. *eumartii* (Carp.) Snyder et Hansen (*F. eumartii*) is a phytopathogenic fungus that causes potato dry rot, one of the most economically important post-harvest diseases of potato tubers. This fungus also produces reddish brown mottling symptoms between leaf veins (Godoy et al., 1996). Despite the economic impact of dry rot, very little is known about how this pathogen causes potato tuber disease. Histological examination indicated that *F. eumartii* hyphae invade the wounded potato parenchyma cells inter as well as intracellularly. The pathogen has been mainly observed into the intercellular spaces, generally in contact with the potato cell walls or growing along them (Godoy, 2000). This type of colonization has also been described for other host plants and *Fusarium* species (Brammall et al., 1988).

In general, filamentous fungi secrete a broad spectrum of proteolytic enzymes during penetration and colonization of the plant tissue. Many of these enzymes

play important roles in nutrition (Dobinson et al., 1997) or in host cell wall degradation (Dow et al., 1998; Carlile et al., 2000). Other roles described for proteases are as toxins, elicitors or in processing zymogens (van den Ackerveken et al., 1993) and inducing necrosis (Paris and Lamattina, 1999). Many species of *Fusarium* produce different proteases in a variety of culture media and host tissues (Urbanek and Yirdaw, 1978; Morita et al., 1994; Griffen et al., 1997). Extracellular proteases produced by *Fusarium* species are involved in the inactivation of enzymes such as cutinases (Murphy et al., 1999). Mosolov et al. (1976) reported a trypsin-like exoprotease produced by *F. solani* in culture medium, which was inhibited by plant-specific proteinase inhibitors. The involvement of proteolytic activity in *Phaseolus vulgaris – Fusarium* spp. interactions was reported by Lange et al. (1996). They described the proteolytic processing of class IV chitinase in the compatible interaction of bean roots with *F. solani* f. sp. *phaseoli*. The accumulation of proteolytic activity in roots infected with this fungus correlated with the processing of class IV chitinase.

Identification and characterization of fungal proteases are prerequisites for understanding their role in the pathogenesis of plant diseases. Previously, the accumulation of an extracellular serine protease in potato tubers after *F. eumartii* infection was described (Olivieri et al., 1998). To get insight into the proteolytic activity involved in *F. eumartii* and potato tuber's interaction, the biochemical characterization of *F. eumartii* extracellular protease is reported. We show that a serine subtilisin protease is able to degrade potato PR proteins as well as specific polypeptides of IWF and cell wall proteins from potato tubers.

Materials and methods

Fungal growth conditions

Fusarium solani (Mart.) Sacc. f. sp. *eumartii* (Carp.) Snyder et Hansen, isolate 3122 (*F. eumartii*) and the non-pathogenic isolate, *Fusarium solani* (isolate 1402), were obtained from the INTA collection, Balcarce, Argentine. The fungi were maintained as stock cultures on potato dextrose agar (PDA) at 4 °C. Small disks of PDA colonized by mycelia were transferred to 250 ml of a liquid medium containing: 1% KNO_3, 0.5% KH_2PO_4, 0.25% $MgSO_4 \cdot 7H_2O$, 0.002% $FeCl_3$ and 1 mg ml^{-1} of denatured potato protein. Cultures were incubated at 25 °C with agitation at 180 rpm for two weeks.

Plant material and preparation of potato-protein extracts

After harvesting, potato tubers were stored in darkness at 4 °C. IWF were extracted (Olivieri et al., 1998) from a 5 day-wounded potato tuber tissue. Wound treatment was made by the hollow punch method (Radtke and Escande, 1973). Proteins from the IWF were concentrated five-fold by precipitation with 4 volumes of cold acetone and resuspended in 50 mM Tris–HCl, pH 8.0, before using as protease substrate. Potato cell wall proteins were extracted according to Isla et al. (1999) with the following modifications. The solid material obtained from the homogenate was resuspended in 50 ml of 1.5 M LiCl and extracted over night at 4 °C. The suspension was centrifuged and the supernatant was dialysed against 20 mM Tris–HCl, pH 8.0. The cell wall extract was concentrated five-fold by acetone precipitation.

Purification of FESP

The culture filtrate (250 ml) was lyophilized, resuspended in 5 ml of buffer 50 mM Tris–HCl, pH 8.0, and applied to a Superose 12 gel filtration chromatography (Pharmacia, FPLC). The running was carried out in 50 mM Tris–HCl, pH 8.0 and 0.5 M NaCl at a flow rate of 0.5 ml min^{-1}. The recovered pool with proteolytic activity was submitted to hydrophobic interaction chromatography on Phenyl Superose HR 5/5 (Pharmacia, FPLC). This column was equilibrated and washed in 50 mM buffer phosphate, pH 7.5, containing 1.7 M $(NH_4)_2SO_4$. The elution was carried out with a linear gradient of 1.7 M $(NH_4)_2SO_4$ to 0.0 M in 20 ml with a flow rate of 0.5 ml min^{-1}. The proteolytic activity eluted with 0.68 M $(NH_4)_2SO_4$. These fractions were concentrated by lyophilization and applied to a Superdex 75 gel filtration chromatography with a flow rate of 1 ml min^{-1} (Pharmacia, FPLC). In this last step, the same buffer and salt concentrations previously described for the Superose 12 column were used. Fractions containing the proteolytic activity were stored at −20 °C for electrophoretical and biochemical characterization.

Enzyme stability was maintained by the addition of 0.5 M NaCl to the running buffer in all gel filtration

chromatographies. This also avoids possible interactions of the enzyme with the column matrices.

N-terminal sequencing of FESP

The purified protease fraction obtained from Superdex 75 chromatography was fractionated in 10% SDS-PAGE (Laemmli, 1970). After separation, proteins were electrophoretically transferred to a PVDF (Inmovilon P^{SQ}) membrane in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) system during 1 h at 100 mA. Transfer buffer was 10 mM CAPS, 20% methanol and 0.02% SDS, pH 11.0. Protein bands were visualized by staining with 0.1% Coomassie blue in 40% methanol. The membrane was destained with 40% methanol and the protein bands were excised. N-terminal sequences were determined by automated Edman's degradation performed with a liquid phase sequence analyser (Model 492, Applied Biosystems). The amino-terminal sequences were submitted to the Swiss Protein Data Bank to determine the relatedness of FESP with other fungal proteases.

Proteolytic activity determinations and protease inhibitor assays

Proteolytic activity was measured in 50 mM Tris–HCl, pH 8.0, using 5 mg of azocasein as substrate in a final volume of 0.5 ml. Reactions were incubated at 42 °C for different times. The reaction was stopped by addition of 0.5 ml 10% TCA, and after 30 min at 40 °C undigested material was removed by centrifugation at 3000 × *g* for 15 min. Activity was estimated as the increase of absorbance at 335 nm of the TCA-soluble fraction. One unit of activity is defined as the amount of enzyme that produces a change of 1 in the absorbance at 335 nm during 1 h at 42 °C. For protease inhibitors studies, each inhibitor indicated in Table 1 was preincubated with protease extracts for 20 min at room temperature, at the concentrations indicated in the same table.

SDS-PAGE analysis

Protein and proteolytic activities were visualized in 12% SDS-PAGE. The protease was revealed with Coomassie Brilliant blue R-250 or with silver nitrate (Blumm et al., 1987). To visualize the proteolytic activity, proteins were dissolved in sample buffer (Laemmli, 1970) without boiling before loading onto the gel. After electrophoresis, the gel was immersed in a solution of 50 mM Tris–HCl, pH 8.0, containing 2% casein. The gel was incubated with agitation during 20 min at 4 °C and then 2 h at 37 °C in the same solution for protein digestion. Areas of protease activity appear as clear bands against the Coomassie-blue stained background.

Table 1. Effect of protease inhibitors on the FESP azocaseinolytic activity. Purified fractions of FESP were incubated with each inhibitor at the indicated concentration. 100% of activity corresponds to 1.0 in absorbance at 335 nm after 1 h of incubation at 42 °C

Inhibitor	Protease specificity	Concentration	Inhibitor activity (%)
None			0
DFP	Serine	2 mM	68
PMSF	Serine	2 mM	65.5
EDTA	Metallo	2 mM	0
E-64	Cystein	1 mM	0
Pepstatin	Aspartic	10 μM	0
Soybean trypsin inhibitor	Trypsin-like	100 μM	0
Aprotinin	Trypsin-like	10 μM	14

Preparations of antigen and anti-protease antiserum

Polyclonal antibodies were raised in a rabbit with subcutaneous injections of the antigen. For antigen preparation, semi-purified protease extract from IWF of potato tubers after 13 days of inoculation with *F. eumartii*, was fractionated on 12% SDS/PAGE. The band corresponding to active serine protease was solubilized in 3 ml of PBS buffer, pH 7.0, and mixed with an equal volume of Freund's complete adjuvant. The second and third injections were prepared in a similar way but using 3 ml of Freund's incomplete adjuvant. Antiserum was collected by cardiac puncture 6 weeks after the booster and was used in Western-blot assay, in a dilution of 1 : 200.

Western-blot assays

Proteins were electrophoresed in 12% or 15% SDS-PAGE, and electro-blotted onto nitrocellulose membrane using a Trans-Blot SD, Semi-Dry Electrophoresis Transfer Cell, 170-3940. After blotting, the nitrocellulose filter was probed with rabbit antiserum and visualized with a second antibody, goat anti-rabbit conjugated with alkaline phosphatase.

In vitro *degradation of potato proteins*

Potato proteins from IWF and cell walls were incubated alone or together with 1.2 U ml^{-1} of *F. eumartii* extracellular protease, during 1 h at 42 °C. The same activity units were used when trypsin (Sigma) was used. When PMSF (2 mM) was used, it was preincubated with the protease during 15 min at room temperature. Incubation mixtures were removed after 1 h, treated with sample buffer and then heated at 100 °C for 3 min before electrophoresis.

Analysis of proteolytic degradation of potato IWF and cell wall proteins were carried out in 15% SDS-PAGE (Laemmli, 1970). The samples containing the reaction mixture, were boiled before electrophoresis. Gels were stained with 40% methanol, 10% acetic acid and 0.25% Coomassie Brilliant blue R-250.

In vitro degradation of potato chitinase and β-1,3-glucanase from IWF of a 5-day wounded tubers was visualized by Western-blot assay. After degradation assays samples were fractionated in 15% SDS-PAGE, blotted onto nitrocellulose membrane and probed individually with potato anti-chitinase and anti-β-1,3-glucanase antisera diluted to 1 : 3000.

Results

Purification and characterization of FESP

Both extracellular serine protease, that accumulated in IWF of potato tubers after challenging by *F. eumartii* and that produced by the fungus in liquid culture medium, share the identical biochemical properties (Olivieri et al., 1998). The fact that this extracellular serine protease might be related with the success colonization of host-plant tissue, leads us to compare the culture filtrate of *F. eumartii* with that of a non-pathogenic strain of *Fusarium solani*, the isolate 1402. The culture filtrate of this last isolate presented very low proteolytic activity (not shown). Moreover, in the IWF extracts from potato tubers inoculated with *F. solani* isolate 1402, which does not cause infection, proteolytic activity was not detected (not shown)

To get a more complete characterization of the serine protease produced during the potato–*F. eumartii* interaction the fungal extracellular serine protease produced in liquid medium was purified and characterized. This extracellular serine protease, named FESP, was purified to apparent homogeneity by successive chromatographies on Superose 12 (not shown), Phenyl Superose, and Superdex 75 columns (Figure 1a and b, respectively). Chromatography on the calibrated Superdex 75 column indicated the FESP native molecular mass was in a range of approximately 25–29 kDa (Figure 1b).

The proteolytic fractions obtained from Superdex 75 chromatography were analysed by electrophoresis (Figure 2). Activity was visualized in SDS caseinolytic gel. The native and denatured protein was revealed by silver staining and Western blot. Analysis of the purified fractions on a caseinolytic gel showed a single activity band of $\geq$66 kDa (Figure 2a, panel I). According to the molecular mass estimated by gel chromatography, the electrophoretic pattern did not truly reflect the protease size. Silver staining identified a single band at the same position, indicating that the protein was purified to apparent homogeneity (Figure 2a, panel II). Moreover, such poor mobility was also revealed when the gels contained lower acrylamide percentages as 7.5% or 10% (not shown). These analyses showed that the purified FESP has a rather slow mobility when it was not boiled. However, under denaturing SDS-PAGE conditions two major polypeptides as well as additional polypeptides of low molecular masses were detected (Figure 2b, panel I). The two polypeptides with apparent molecular masses between 30–33 kDa, were recognized by the polyclonal antibodies raised against the serine protease from IWFs of infected potato tubers (Figure 2b, panel III). They correspond to the molecular masses estimated by gel filtration chromatography. Besides, both polypeptides lack of detectable proteolytic activity in this condition (not shown).

The purified protease was employed to determine some biochemical properties. The optimal pH for the hydrolysis of azocasein had a wide range between pH 4.0 and 9.0, having a maximum at pH 8.0. The activity was not affected either by change in the ionic strength between 0.02 and 1.7 M or by addition of SDS in increasing concentrations just to 2.5%. To show whether this enzyme is glycosylated, glycoprotein affinity chromatographies, phenyl-boronate and concanavalin A, were performed. Proteolytic activity did not bind to these matrixes suggesting that FESP is not glycosylated (not shown).

Effect of protease inhibitors

Previous results indicated that the proteolytic activity bands detected in the *F. eumartii* culture filtrate by SDS caseinolytic gel were inhibited by 2 mM DFP and 2 mM PMSF (Olivieri et al., 1998). To have a complete characterization of FESP, the pure protease was classified

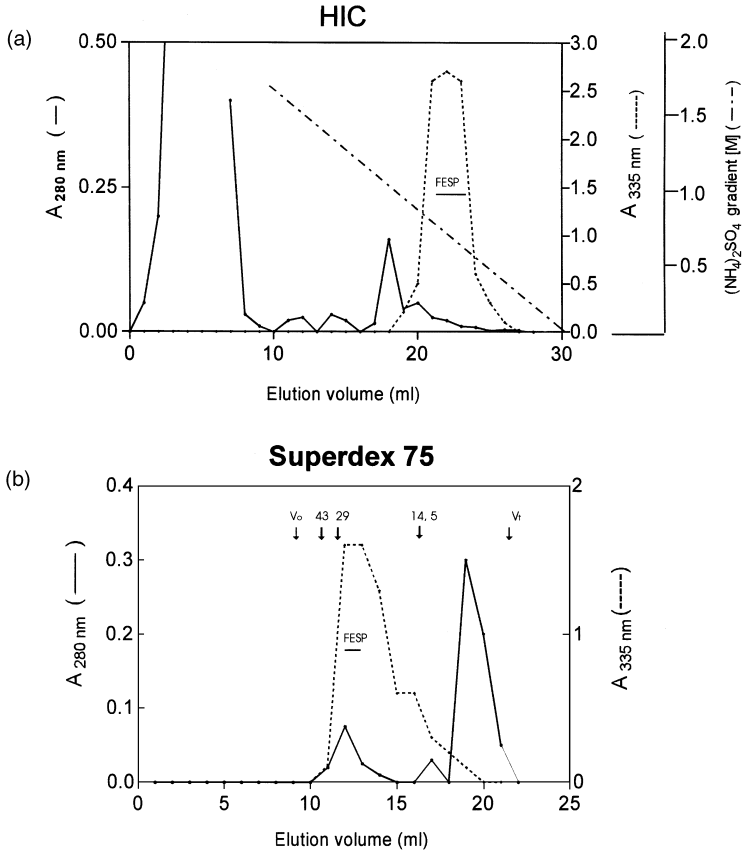

Figure 1. FPLC elution profile of FESP. (a) Hydrophobic interaction chromatography (HIC) in Phenyl Superose column. Gel filtration fractionated culture filtrate was applied to HIC and eluted with a decreasing gradient of $(NH_4)_2SO_4$. (b) Gel filtration chromatography in Superdex 75. The pool recovered from Phenyl Superose containing proteolytic activity was concentrated and submitted to Superdex 75, in the conditions described in Materials and methods. Proteolytic activity was determined with azocasein (A_{335nm}) as described in Materials and methods.

with the use of standard inhibitors (Table 1). As is shown in Table 1, DFP and PMSF inhibited the azocaseinolytic activity, 68% and 65%, respectively, indicating that FESP is a serine protease. No inhibition was detected for the cystein, metallo- and aspartil-protease inhibitors, nor for those of trypsin like proteases, as soybean trypsin inhibitor and aprotinin.

N-terminal amino acid sequence comparison

The N-terminal amino acid sequences of Mr 30 and 33 kDa proteins recovered from the purified and denatured FESP, (indicated by arrows in Figure 2b panel I) was determined. The sequence of the first 13 residues was obtained from the 30 kDa polypeptide (Figure 3). Comparisons of the FESP N-terminal sequence to polypeptides in the SwissProt data Bank showed that this region has a high degree of sequence similarity to subtilisin-related serine proteases. The highest similarity found was with the N-terminal of the mature alkaline protease from *Trichoderma harzianum* (Geremía et al., 1993) and with the following sequences: Alp2 from *Cochliobolus carbonum* (Murphy and Walton, 1996), Alp from *Aspergillus oryzae* (Tatsumi et al., 1989), Alp from *Aspergillus fumigatus* (Jaton-Ogay et al., 1992); Alp from *Aspergillus flavus* (Ramesh et al., 1994); and Proteinase K from *Tritirachium album* (Gunkel and Gassen, 1989). This analysis showed the presence of six invariant residues in all the fungal sequences included (–TTQ–A–GL). However, some residues were distinct when FESP was compared to the five fungal proteinases (Figure 3). These data indicated that FESP is a new member of subtilisin-related alkaline protease.

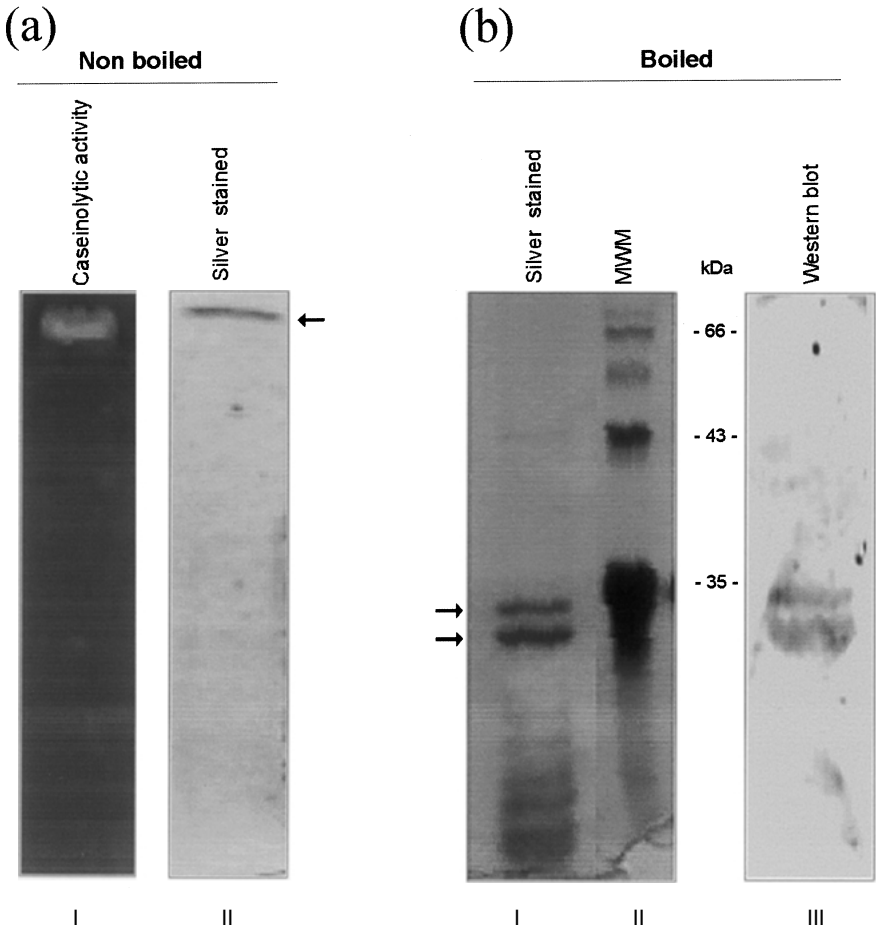

Figure 2. Electrophoretic patterns of FESP. Purified fraction of FESP obtained from Superdex 75 chromatography was analysed in 12% SDS-PAGE. (a) Electrophoretic behaviour under non boiling condition. Proteolytic activity of FESP after incubation the gel in 2% casein solution and Coomassie blue staining (panel I), and silver staining (panel II). (b) Electrophoretic behaviour of FESP after boiling, visualized by silver staining (panel I). Molecular weight markers (MWM) are visualized by silver staining in panel II. Immunodetection with anti-IWF protease serum (panel III). I. Arrows indicated the bands recognized by the anti-IWF protease serum.

Figure 3. N-terminal amino acid sequence comparison of FESP with other fungal proteases. Shaded areas indicate conserved amino acids among proteins. *Trichoderma harzianum* (Geremía et al., 1993); *Cochliobolus carbonum* (Murphy and Walton, 1996); *Aspergillus oryzae* (Tatsumi et al., 1989); *Aspergillus fumigatus* (Jaton-Ogay et al., 1992); *Aspergillus flavus* (Ramesh et al., 1994); *Tritirachium album* (Gunkel and Gassen, 1989).

All mentioned enzymes are alkaline proteases that contain a proregion and a signal peptide. As in the case of FESP polypeptide, the molecular masses of these mature proteins are in the range 28–41 kDa.

The sequence of the first eight residues from the lower mobility band (33 kDa) was LPDISYFN. This polypeptide has an N-terminal sequence with no similarity to any sequence in the non-redundant databases. A comparison of this sequence with the deduced amino acid sequences of enzyme proregions from different genus of fungi shown in Figure 3 did not indicate similarities.

In vitro *degradation of potato extracellular proteins by FESP*

F. eumartii has a high specificity to penetrate through wounded sites and colonize potato tuber tissue by the intercellular spaces and across the cell wall (Godoy, 2000). In consequence, the action of FESP on potato extracellular proteins was investigated. FESP was incubated with two extracellular fractions of a 5-day wounded potato tuber tissue: IWF extracts and cell wall proteins. In these two fractions, proteolytic activity was not detected (not shown). The results of these assays were visualized by SDS-PAGE and Western-blot assays (Figure 4). The degradation pattern of IWF proteins from wounded tubers is shown in Figure 4a. Degradation of at least three polypeptides of Mr 22–25 kDa and Mr 43 kDa was detected (Figure 4a, lane 4). This degradation was inhibited by PMSF (Figure 4a, lane 5). In contrast, an abundant polypeptide of faster electrophoretic mobility of approximately 16 kDa, remained undegraded after incubation.

Assuming that potato cell wall proteins may also be susceptible to fungal hydrolytic enzymes the cell wall proteins were incubated in the same conditions that IWF extracts (Figure 4b) and a similar degradation pattern were obtained (Figure 4b). Subsequently, antichitinse and antiglucanase antibodies were used to determine the PR protein levels in potato extracts. Although, basic chitinase and β-1,3-glucanase were detected in IWF from wounded tubers, they were not detected in cell wall extracts (not shown). To test whether these PR proteins were degraded by FESP, a Western-blot analysis was performed with anti-chitinase and anti-β-1,3-glucanase sera (Figure 4c). Two chitinase bands (32–35 kDa) and a single glucanase band (32 kDa) were revealed by the sera (Figure 4c). Both enzymes were degraded

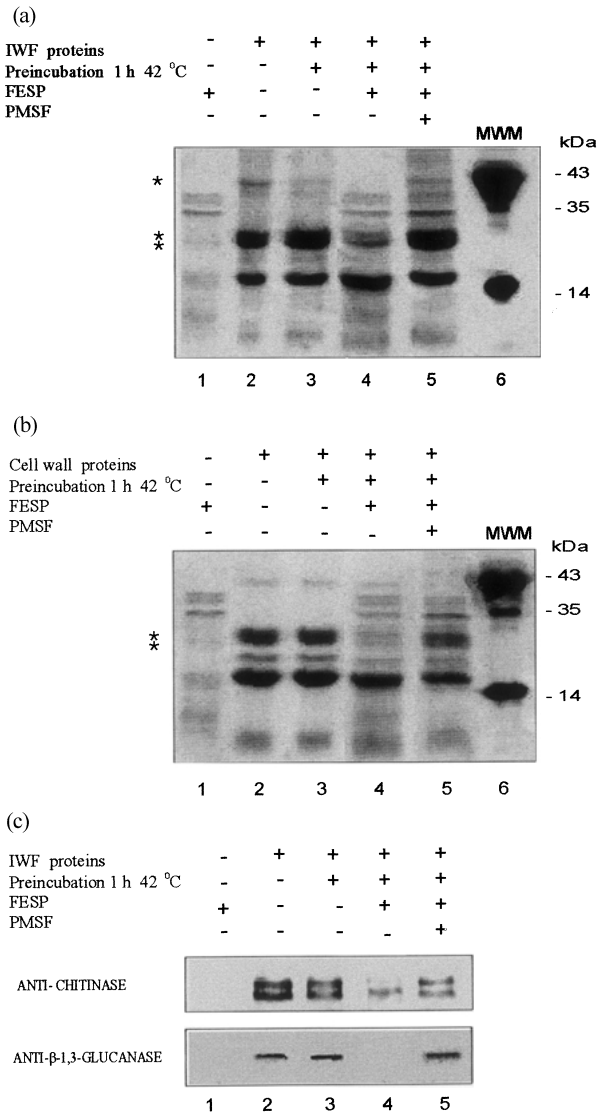

Figure 4. In vitro degradation of potato proteins by FESP. Aliquots of IWF extracts (a) and (c), or cell wall proteins (b) were individually incubated alone or with FESP at 42 °C for 1 h and then analysed by SDS-PAGE and Western blot. Gels shown in (a) and (b) were stained with Coomassie blue. The same extracts incubated in (a) were transferred onto nitrocellulose and revealed with anti-chitinase or anti-glucanase antibodies (c). Asterisks indicate the bands that were degraded by FESP. The different treatments are indicate in the figure. MWM, indicate the molecular weight markers.

after incubating with FESP (Figure 4c, lane 4). Again this degradation was inhibited by preincubation of the enzyme with PMSF (Figure 4c, lane 5). Since the PR proteins are known to be relatively resistant to

proteolysis and, in order to test the specificity of FESP for potato proteins, the fraction containing PR proteins was incubated with commercial trypsin: no degradation was observed (not shown).

Discussion

A FESP was purified to apparent homogenity. This was characterized as a serine protease belonging to subtilisin family. It comprised the majority of the *F. eumartii* extracellular proteolytic activity detected in culture filtrates. According to the reported purification procedure, no evidence for additional proteases was found. However, the possibility that *F. eumartii* produces other proteases in liquid culture cannot be ruled out. The fact that the infection of potato tubers with *F. eumartii* was accompanied with the accumulation of a serine-protease activity (Olivieri et al., 1998), and that, it was also present in the culture filtrate of this fungus, lead us to suggest that this serine-protease activity might be involved in host-plant colonization. This hypothesis was supported by data that indicate that this activity was present neither in healthy tubers, nor in the culture filtrate of the non-pathogenic relative, *Fusarium solani* isolate 1402, nor in IWF extracts obtained from potato tubers after challenging with this non-pathogenic isolate (not shown).

The N-terminal amino acid sequence of the approximately 30 kDa polypeptide obtained from denatured FESP show high identity with those of mature serine protease subtilisin family from different fungi (Geremia et al., 1993; Jaton-Ogay et al., 1992; Tatsumi et al., 1989). On the other hand, the data bank search showed that the N-terminal amino acid sequence of the 33 kDa polypeptide did not show similarities with other known proteins. Most of the previously mentioned reports of fungal alkaline proteases describe a putative precursor and mature form for the subtilisins. In addition, it is also known that there is more sequence variation in the pre- and prosequence regions than in the mature regions. It is therefore suggested suggest that the 33 kDa polypeptide may be the precursor of FESP, but it will be necessary to clone the gene to confirm the hypothesis. The action of the protease inhibitor on the purified FESP was consistent with the classification of FESP as a subtilisin serine protease. Extracellular subtilisin proteases have been reported to participate in the pathogenicity of different system (Reddy et al., 1996). Expression of subtilisin-like proteases may have a wide distribution, including phytopathogens, mycopathogens, saprophytes, etc. (Reddy et al., 1996; Leger et al., 1992).

SDS-PAGE analysis indicated that FESP migrates as a considerably larger protein under non-denatured conditions, independently of acrylamide concentrations. The hydrophobic characteristic associated with the native FESP, as judged by its binding to hydrophobic interaction chromatography, might be one of the causes of the atypical electrophoretic pattern. Another cause may be glycosylation (Lindstrom et al., 1993). However, there is no evidence that FESP is a glycosilated protein. Tatsumi et al. (1989) also reported a non-glycosilated fungal alkaline proteases. Abraham and Breuil (1996) described a subtilisin-like serine proteinase secreted by *Ophiostoma piceae*. This proteinase, as well as FESP, was purified from culture filtrate by hydrophobic interaction chromatography. Both enzymes were susceptible to autolytic degradation during chromatographic separations when salt was not present. Moreover, this feature is also similar to that reported for the bacterial subtilisin (Owers Narhi and Arakawa, 1989), and for the fungal serine protease At1 of *Acremonium typhinum* (Lindstrom and Belanger, 1994). Since FESP belongs to the subtilisin protease familiy, it might also share the anomalous electrophoretic behaviour reported for the subtilisin upon SDS-PAGE. In this case, the native protease (folded) runs near the top of the gel. In contrast, the denatured and unfolded form migrates farther down the gel according to the expected molecular mass (Owers Narhi and Arakawa, 1989). Heating of FESP in the presence of SDS prior to electrophoresis resulted in two major and other minor bands. Two major bands are near to the molecular mass range estimated by gel filtration chromatography. In addition, bands of low molecular masses may correspond to minor degradation products. They probably come from autodigestion which is a common effect associated to several proteases. Moreover, for the case of aprA-subtilisin, autolysis occurs even in the presence of PMSF (Owers Narhi and Arakawa, 1989). Since the Western-blot assays show that the anti-protease serum recognized these two denatured bands, it appears that they both belong to the same protease.

In addition, it has been assumed that fungal hydrolytic enzymes might be involved in the degradation of host extracellular matrix and apoplastic contents during invasion (Rogers et al., 2000). This prompted us to analyse the action of FESP on IWF extracts and cell wall proteins. Since in field conditions *Fusarium* species naturally requires wounds to infect potato

tubers, we confronted FESP with potato cell walls and IWF extracts from wounded tubers. Coomassie blue patterns of these extracellular extracts were similar, either after or before incubation with FESP.

Although, we cannot assume that bands which have the same electrophoretic mobility correspond to the same proteins, it is likely that both compartments share some proteins. Nevertheless, extracts were differentially enriched with extracellular proteins because basic chitinases and 1,3-glucanases were only immunodetected in the IWF extracts from wounded tubers and not in cell wall extracts. After incubating the potato cell wall proteins with FESP, specific proteins between 20 and 25 kDa were clearly degraded. Unfortunately, there is little information on potato cell proteins, so it will be of interest to identify the degraded proteins and analyse their biological functions. Interestingly, Western-blot assays show that, *in vitro*, FESP was able to degrade chitinases and a β-1,3-glucanase accumulated in the IWF of wounded tubers. These isoforms possess similar antigenic sites that those described by Kombrink et al. (1988) which are increased in IWF of potato leaves inoculated with *Phytophthora infestans*.

Ancillo et al. (1999) showed that two isoforms of chitinases with similar electrophoretic mobility were detectable in potato tubers. Proteolytic processing and/or degradation of PR proteins by proteases may occur. Rodrigo et al. (1989) reported that a plant constitutive aspartyl endoproteinase degrades host PR proteins present in IWF of tomato leaves. More recently, Dow et al. (1998) described a bacterium metalloprotease that degrades PR proteins from *Brassica campestris*. The importance of proteases as virulence factors remains unclear because in many cases, the deletion, mutation or disruption of proteases did not affect the pathogenicity (Jaton-Ogay et al., 1994). The ability of *F. eumartii* to degrade potato PR proteins may be related with the fungal colonisation of potato tissue. Previous results reported by Godoy et al. (1996) indicated that a basic β-1,3-glucanase of potato plantlets may have antifungal properties against *F. eumartii*.

Finally, it is premature to ascribe the precise significance of FESP in potato dry rot. However, the data provided here might be of importance for further studies aimed to counteract the successful colonisation of potato tubers by *F. eumartii*. The available N-terminal amino acid sequence information will help us to clone the FESP gene and provide the molecular tools required to determine its role in potato–*F. eumartii* interaction.

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