CMLS, Cell. Mol. Life Sci. 60 (2003) 1470–1476
1420-682X/03/071470-07
DOI 10.1007/s00018-003-3079-7


CMLS Cellular and Molecular Life Sciences

## Research Article

# PcF protein from *Phytophthora cactorum* and its recombinant homologue elicit phenylalanine ammonia lyase activation in tomato

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Received 22 February 2003; received after revision 25 March 2003; accepted 14 April 2003

**Abstract.** The phytotoxic protein PcF (*Phytophthora cactorum-Fragaria*) is a 5.6-kDa cysteine-rich, hydroxyproline-containing protein that is secreted in limited amounts by *P. cactorum*, an oomycete pathogen of tomato, strawberry and other relevant crop plants. Although we have shown that pure PcF triggers plant reactivity, its mechanism of action is not yet understood. Here we show that PcF, like other known fungal protein elicitors involved in pathogen-plant interaction, stimulates the activity of the defense enzyme phenylalanine ammonia lyase (EC 4.3.1.5) in tomato seedlings. Recognizing that a key step in understanding the mechanism of action of PcF at a molecular level is knowledge of its three-dimensional structure, we overexpressed this protein extracellularly in *Pichia pastoris*. The preliminary structural and functional characterization of a recombinant PcF homologue, N4-rPcF, is reported. Interestingly, although N4-rPcF is devoid of proline hydroxylation and has four additional amino acid residues attached to its N terminus, its secondary structure and biological activity are indistinguishable from wild-type PcF.

**Key words.** *Phytophthora cactorum*; phytotoxic protein; hydroxyproline; circular dichroism; eliciting activity; phenylalanine ammonia lyase activation.

Analysis of the effects exerted on the metabolic profile of plants by pure phytotoxic proteins secreted by plant pathogens provides useful information on the signaling mechanisms underlying the outcome of pathogen attack, i.e. either resistance or disease [1]. Such investigations have allowed functional characterization of several proteins from fungal plant pathogens, e.g. the elicitins [2] and other avirulence protein families [3].

Recently, the *Phytophthora cactorum-Fragaria* (PcF) protein, a novel phytotoxic factor secreted at low levels by cultured *P. cactorum*, has been isolated and characterized, including cloning and sequencing of the full-length cDNA [4]. Mature PcF, secreted after N-terminal signal processing, is an acidic 52-amino acid protein comprising a 4-hydroxyproline at position 49, and six cysteine residues bridged intramolecularly. According to the criteria proposed by Templeton et al. [5], PcF might be related to the small, cysteine-rich fungal proteins involved in plant-pathogen interaction. A homology search in the sequence databases did not provide any clues on PcF function, but revealed homology with an unknown protein from *P. infestans* (GenBank Acc. BE775988) [6]. As proposed by van den Hooven et al. [7], we believe that

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knowledge of the three-dimensional fold and S-S bridging pattern of PcF will enable a more refined analysis of structure and function that will provide important information about the physiological role(s) of this protein.

*P. cactorum* is an oomycete phytopathogenic toward a wide range of host plants, including strawberry and tomato [8]. Though we have shown that treatment with pure PcF causes leaf-withering symptoms in both plant species [4], the underlying molecular mechanism for this remains to be investigated in detail. Nonetheless, our previous data indicate that the unfolded $\beta$-propionamidated PcF is not able to trigger tomato seedling withering, thus suggesting a correlation between the three-dimensional structure of this protein and its biological function [4]. In addition, since very little is known about the biological role of PcF, we envisaged that evaluation of the induction of the plant defense marker enzyme phenylalanine ammonia lyase (PAL) might provide useful information. In fact, various protein elicitors are known to stimulate, in cultured plant cells, both PAL enzyme activity [9, 10] and PAL gene expression [11]. PAL (EC 4.3.1.5) catalyzes the conversion of L-phenylalanine to trans-cinnamic acid, which is a precursor for a variety of defense- and wound-related compounds [9, 12]. Since PAL activation is related to a distinct signal transduction pathway with respect to oxidative burst and apoptotic cell death [11], PAL induction by a pathogen-derived elicitor could provide important insights into the signal transduction mechanism that triggers the plant response. Furthermore, the extent of PAL stimulation might enable a quantitative evaluation of both plant responsiveness and protein effectiveness.

Realizing that a detailed structural and functional analysis will require significant amounts of protein, we overexpressed PcF extracellularly in *Pichia pastoris,* using a strategy based on integration of the foreign gene at the alcohol oxidase 1 *AOX1* locus and on the methanol-regulated induction of protein synthesis [13, 14].

Here we present data showing that wild-type PcF, when tested on tomato seedlings, not only induces leaf withering [4] but also elicits PAL activation. Additionally, we report the cloning, overexpression and purification of a PcF homologue that has four extra amino acid residues at its N terminus and is devoid of 4-hydroxyproline. To obtain a preliminary evaluation of both functional and secondary-structure homology between the recombinant and wild-type proteins, we compared both their PAL-inducing activity and circular dichroism (CD) spectra.

## Materials and methods

### Strains and growth

The *P. cactorum* P381 strain, kindly provided by Prof. G. Cristinzio (University of Naples 'Federico II,' Italy), was grown as previously reported [4]. DNA subcloning was completed in *Escherichia coli* Top10F′ cells (Novagen) [15]. *P. pastoris* transformation and protein expression were carried out in the histidine-requiring auxotroph GS115 strain (Invitrogen), by growing in minimal dextrose (MD) plates, minimal methanol (MM) plates or medium, and minimal glycerol (MGY) medium [16].

### Cloning strategy and heterologous expression in *P. pastoris*

Extracellular expression in *P. pastoris* was carried out according to the *Pichia* Expression Kit [16], by using the pPIC9 expression vector (Invitrogen) appropriately endowed with the yeast secretion signal $\alpha$-mating factor [17]. For this reason, a PcF-coding sequence lacking the native secretion signal was isolated by genomic PCR, following total DNA purification from a P381 *P. cactorum* mycelium homogenate [18], and ligated into the pPIC9 vector at the *Eco*RI site [15]. The primers oligo fw and oligo rev (fig. 1) were designed on the basis of the PcF cDNA sequence (GenBank Acc. AF354650). In the resulting pPIC9-PcF vector, correct insert orientation and sequence were checked by automated sequencing [19]. Owing to this cloning strategy, four extra amino acid residues, YVEF, are expected at the N terminus of the recombinant protein (fig. 1).

*P. pastoris* GS115 cells were transformed by electroporation using 20 µg of the *Bgl*II-linearized pPIC9-PcF vector, and the transformant clones were selected by their reduced growth on methanol versus dextrose medium. Genomic integration of the PcF expression cassette in the yeast genome was further checked by colony PCR [20], performed on the lyticase-treated yeast cell suspensions, with the primers 3′ and 5′AOX1 [16]. Protein expression was performed by growing a PCR-positive clone in 1 l MGY medium up to an $OD_{600}$ of 4 (about $2.0 \times 10^8$ cells/ml), then by resuspending the cells in 200 ml MM medium for growth induction, with daily methanol pulses [16]. Under these conditions, the expressed protein is secreted in the culture medium. The time-course of protein secretion was monitored, on culture medium aliquots collected at different induction times, by tricine-SDS-PAGE [4, 21] as well as by C18 HPLC recording the single $OD_{230}$ peak area (see below). After reaching the maximum $OD_{230}$, the culture medium was collected by centrifugation (4000 g, 10 min), and lyophilized.

### Protein purification and characterization

Wild-type PcF was purified to homogeneity from the *P. cactorum* culture filtrate as previously reported [4], whereas, to obtain pure N4-rPcF from the *P. pastoris* expression culture medium, the last step in the above procedure was sufficient. Briefly, the lyophilized supernatant from 200 ml of expression culture was resuspended in 5 ml 0.1% trifluoroacetic acid (TFA) and loaded in 1-ml

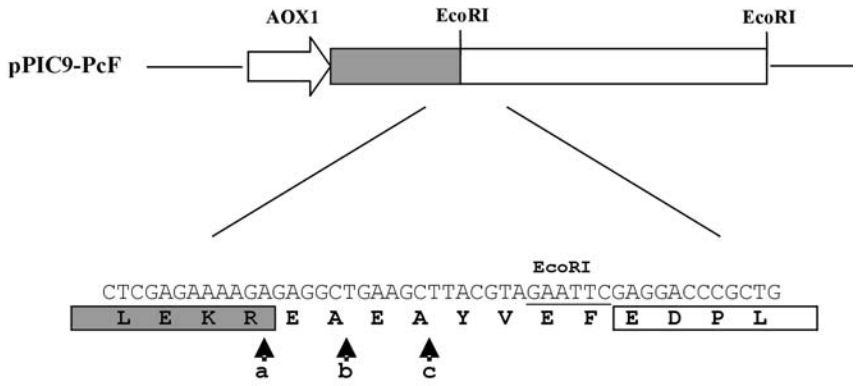


Figure 1. Cloning site of the expression vector pPIC9-PcF, and predicted N terminus of the PcF homologue expressed in *P. pastoris*. The PCR-based cloning was obtained using the primers 5′-ATATAGAATTCGAGGACCCGCTGTACTGCCAG-3′ (oligo fw) and 5′-TAATGAATTC**TTA**CTACGCGGAAGCTGGAGTGGT-3′ (oligo rev), both incorporating *Eco*RI sites in the non-annealing region (underlined), and, in the reverse primer, an additional stop codon (bold) to ensure efficient termination. The shaded sequence depicts the end of the $\alpha$ factor signal peptide, while the boxed one indicates the start of mature PcF from *P. cactorum*. Arrowheads (a–c) indicate the multiple processing sites in the expressed pre-protein precursors [17]. YVEF represents the four extra amino acid tag expected at the N terminus of the recombinant protein.

aliquots on C18 HPLC [4]. The eluted fractions active in the tomato seedlings bioassay (see below), corresponding to a single $OD_{230}$ peak, were pooled and lyophilized. Automated Edman sequencing was performed on a Procise Model 491 gas-phase sequencer (Applied Biosystems, Foster City, Calif.). Mass determinations were performed by liquid chromatography/mass spectrometry (LC/MS) on an integrated Agilent (Palo Alto, Calif.) 1100 system. The pure N4-rPcF protein concentration was evaluated spectrophotometrically by using the $\varepsilon_{280}$ 4845 $M^{-1}$ $cm^{-1}$ calculated from its amino acid composition [22].

**Biological activity assays**

The phytotoxic activity of either crude or purified protein fractions was routinely assayed on tomato seedlings, previously chosen as a selective model system for testing PcF bioactivity [4]. Accordingly, the PAL-eliciting ability of PcF and N4-rPcF was also tested on the same tomato system. Sets of five root-excised tomato seedlings were incubated in triplicate eppendorf tubes containing 2 µg of pure protein in 100 µl distilled water; triplicate sets of controls, comprising five root-excised tomato seedlings in distilled water, were run in parallel. At set time intervals, sets of five seedlings, either treated or controls, from individual eppendorf tubes (about 0.1 g total weight) were harvested, mixed with 0.04 g sterile sand in 200 µl of 50 mM Tris/HCl buffer, pH 8.5, containing 14 mM 2-mercaptoethanol, 5 mM 1,10-phenanthroline, 1 mM phenylmethylsulfonylfluoride (PMSF), and 1.5% (w/v) polyvinylpyrrolidone (PVP), and immediately pestle-homogenized to obtain crude extracts. To evaluate PAL activity, the crude extracts were clarified by centrifugation at 16,000 g for 30 min, and 100-µl aliquots were incubated in parallel at 30 °C in a 300-µl reaction mixture containing 4 mM phenylalanine substrate, in 50 mM Tris/HCl buffer, pH 8.5. After 90 min incubation, the reaction was stopped by adding acetic acid to 5% (v/v), and the trans-cinnamic acid formed was quantified by C18 HPLC (ODS Ultrasphere, 4.6 × 250 mm, Beckman), using a 50% methanol, 0.1% acetic acid isocratic elution, and authentic trans-cinnamic acid (Sigma) as the standard. Protein concentrations were determined by the Bio-Rad protein assay [23]. PAL specific activity was then derived from these data. Based on this experimental protocol, each data point represents the mean of three experiments carried out using five pooled, treated or untreated, seedlings. For each time point, the PAL values from the treated samples were compared to those from the corresponding untreated samples, using the Student unpaired t test for unequal variances. The statistical analysis was carried out by using the Microsoft Excel version 2002 program.

**CD measurements**

CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier system PTC-348 WVI for temperature control. The spectra were collected at 20 ± 0.1 °C; the spectral range was 260–186 nm, using a 2-mm path length cell and a $6 \times 10^{-6}$ M protein concentration. Data are the average of five separate runs, and the ellipticity is reported as the mean residue molar ellipticity, [θ], (deg $cm^2$ $dmol^{-1}$).

## Results and discussion

**N4-rPcF overexpression in *P. pastoris* and purification**

According to the experimental protocol described in Materials and methods, an appropriate PcF-coding DNA

fragment was initially isolated directly from the *P. cactorum* genome. The determined nucleotide sequence of this fragment exactly matched the previously isolated cDNA [4], revealing the absence of introns. Following *P. pastoris* transformation with the pPIC9-PcF expression vector, four *AOX1*-deficient transformant clones were isolated. Genomic analysis revealed that the pPIC9-PcF integration cassette had replaced the wild-type *AOX1* locus, with a single omega insertion event (fig. 2A). Clone 4 was used for the large-scale expression culturing. Aliquots of the culture medium collected at time intervals during an induction period of 6 days, showed a single band corresponding to a 6.2-kDa protein on SDS-PAGE (not shown), and, upon C18 HPLC, a single peak absorbing at 230 nm (fig. 2B), both increasing in an induction-time-dependent fashion. In addition, the bioassay on tomato seedlings revealed that the entire toxic activity in the crude *P. pastoris* supernatant co-eluted with this $OD_{230}$ HPLC peak. Since the protein secretion leveled off after 6 days of culturing (fig. 2B, inset), this time was selected as the optimal one. After preparative C18 HPLC, about 150 mg of pure recombinant protein per liter of expression culture were obtained. This amount is at least three orders of magnitude higher than

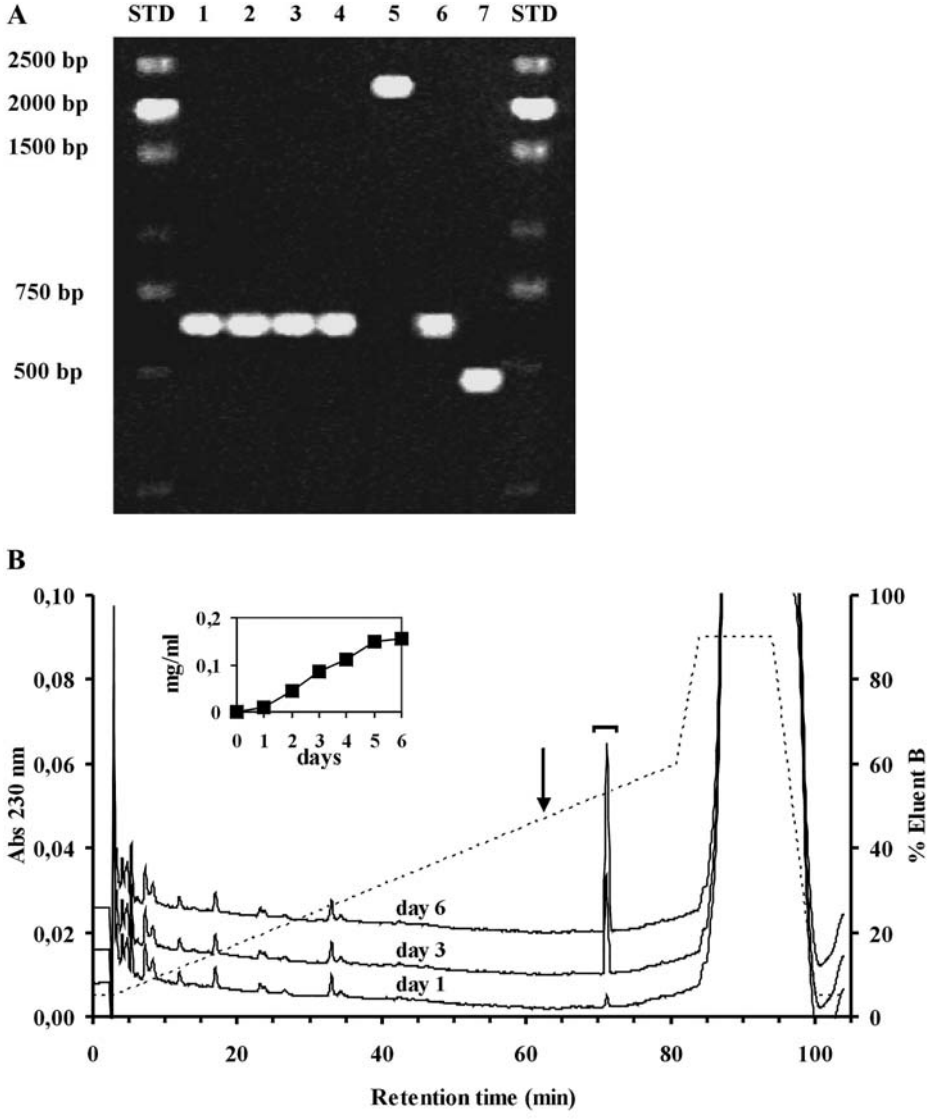


Figure 2. (*A*) Genomic PCR amplifications of the recombinant *AOX1* loci in *P. pastoris* transformants. Lanes 1–4, four transformant clones; lane 5, the 2.2-kb wild-type *AOX1* locus; lane 6, the 660-bp PcF expression cassette in the pPIC9-PcF vector; lane 7, the 492-bp empty cassette in the original pPIC9 vector. (*B*) C18 HPLC pattern of the expression culture medium (150-µl samples) of the above clone 4, at different periods of induction. Broken line, the gradient obtained with buffer A (0.1% TFA) and B (0.1% TFA, 65% acetonitrile). The downward square bracket delimits the biologically active fractions, overlapping the N4-rPcF $OD_{230}$ peak. The arrow marks the expected retention time of the wild-type PcF protein under the same chromatographic conditions [5]. Inset: time-course of N4-rPcF protein secretion in the culture medium.

the yield of wild-type PcF from *P. cactorum* per liter of culture filtrate [4].

**N4-rPcF molecular features**

The partial N-terminal sequencing showed the individual sequence YVEFEDPLY…, consistent with a homogeneous recombinant PcF protein originating from successful $\alpha$ factor processing by the *P. pastoris* secretion machinery [17]. LC/MS analysis confirmed the presence of a single molecular species of 6144 ± 0.5 Da, in agreement with the expected mass of a YVEF-tagged PcF protein, with three intramolecular S-S bridges and devoid of post-translational modifications, e.g. proline hydroxylation. In accordance with the hydrophobic contribution of these alterations, the C18 HPLC retention time of the recombinant protein peak turns out to be slightly higher than for wild-type PcF (fig. 2B). To denote the presence of the four extra N-terminal residues we named the recombinant protein N4-rPcF.

**Comparative functional characterization**

*1) Withering of tomato seedlings.* About 2 µg of pure N4-rPcF protein is the minimal amount required to achieve tomato seedling withering within 20–36 h, in perfect agreement with the previously reported degree of effectiveness of the wild-type PcF protein [4] and consistent with the level of activity of other known fungal proteins [24–26]. Interestingly, as previously described for the wild-type PcF [4], heat-treated N4-rPcF (100 °C for 5 min) maintains full activity, while it is completely inactivated following $\beta$-propionamidation (data not shown). These findings suggest that both protein forms possess a thermostable fold and that intact disulfide bridges are required for biological activity.

*2) PAL elicitation.* Preliminary experiments revealed that about 0.1 µg of either recombinant or wild-type PcF (corresponding to a protein concentration of about 170 nM, under the assay conditions described in Materials and methods) were sufficient to detect significant PAL activation (data not shown). However, since we were interested in studying plant withering and PAL activation simultaneously, we chose to treat tomato seedlings with 2 µg of either PcF (3.6 µM) or N4-rPcF (3.3 µM). As shown in figure 3, both proteins triggered a statistically significant PAL stimulation over the controls at each time point ($p < 0.05$, $t > 2.23$), with the same time-course, i.e. a tenfold peak at 3 h, followed by an average sixfold activation plateau. Interestingly, the timing of peak activation (3 h) is consistent with previous observations in elicitin-treated cultured tobacco cells [9, 11]. However, in our whole-plant system, the PAL activation level remained high during the withering process, in agreement with findings by other authors reporting that PAL induction proceeds even after the initiation of the elicitor-induced plant cell death process [10, 27]. These results indicate that both PcF and N4-rPcF are perceived in the same manner by the host plant and appear to be functionally indistinguishable. Finally, the low PAL activation exhibited by the untreated controls, which can be ascribed to the root-cutting injury itself [12], does not alter the significance of the functional comparison between the two protein homologues.

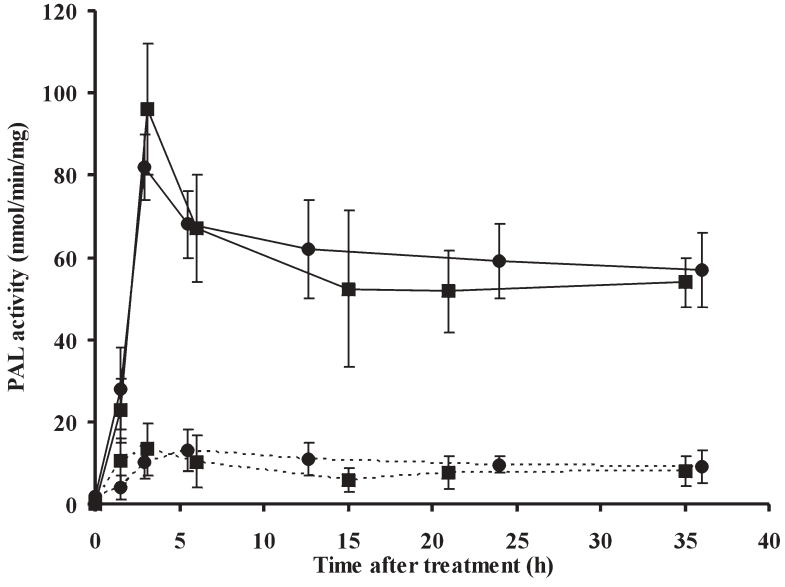


Figure 3. Stimulation of PAL enzyme activity in tomato seedlings in response to pure wild-type PcF and N4-rPcF proteins. Closed circles indicate PAL specific activity at different times of treatment with PcF (continuous line), and the corresponding untreated controls (dotted line). Closed squares refer to seedlings treated with N4-rPcF (continuous line) and the corresponding untreated controls (dotted line). Each time point represents the mean ± SD of three independent experiments. Withering symptoms were observed in all protein-treated tomato seedlings within 20–36 h, while controls remained fully viable.

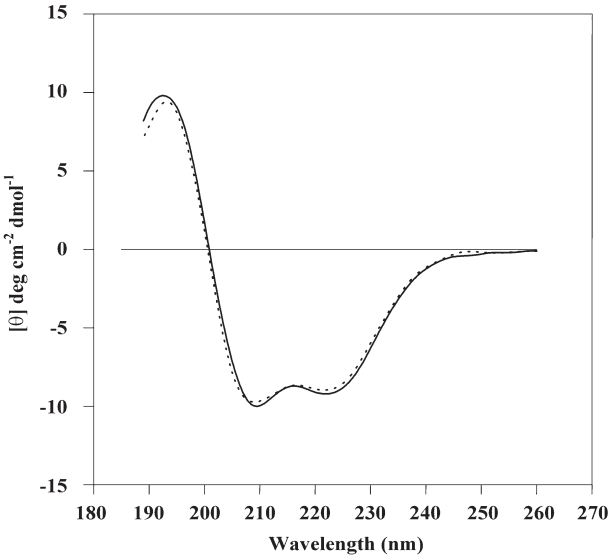


Figure 4. Far-UV CD spectra of wild-type PcF (continuous line) and N4-rPcF (dotted line) proteins, in 3 mM potassium phosphate, pH 5.8, at 20 °C.

## Conformational analysis by CD

As shown in figure 4, N4-rPcF exhibits a CD spectrum indistinguishable from the wild-type PcF protein, suggesting that their secondary structures are quite similar. The position of the optical bands at 222, 209 and 193 nm are indicative of the presence mainly of $\alpha$-helical secondary-structure elements. However, deconvolution of the spectra carried out using convex constrained analysis (CCA) [28] indicates the presence of only 25% $\alpha$ helix, with 58% of $\beta$ components, combining $\beta$ strands and $\beta$ turns. Of the remaining 17%, 13% is identified to be random and 4% due to aromatic and/or S-S contributions. Clearly, only the determination of the protein three-dimensional structure will clarify this discrepancy.

## Conclusions

The present report shows, for the first time, that pure wild-type PcF is able to trigger in tomato seedlings a typical defense reaction, namely PAL activation, a functional feature common to other protein elicitors [9–12]. Aware that further evidence is needed to establish whether PcF can be considered a true elicitor, and to assess its role in the process of plant-pathogen interaction, we have devised a protocol to produce suitable amounts of a recombinant form of PcF.

The analysis of the primary structure revealed that the recombinant protein, N4-rPcF, comprises an additional YVEF N-terminal tag and lacks hydroxylation in Pro49. As for its structural features, CD spectroscopy showed that N4-rPcF exhibits a CD spectrum superimposable on that of PcF, thus suggesting an equivalent secondary structure. The evaluation of its biological activity showed that, like the wild-type form, it induces withering in tomato seedlings, and elicits an equivalent PAL activation pattern as well.

Together, these results indicate that the additional YVEF N-terminal tag and the lack of Pro49 hydroxylation do not alter significantly either the biological activity or the secondary structure of the recombinant protein, with respect to the wild-type one. Moreover, the loss of activity observed for both PcF and N4-rPcF in their unfolded cysteine-alkylated state is evidence for a clear structure-function correlation, and enforces the need for the elucidation of its three-dimensional structure. This is currently underway in our laboratory.

Finally, even though we have not investigated whether PcF triggers other signal transduction pathways, PAL activation singles out a PcF-responsive pathway in tomato which offers a tool to measure the bioactivity of PcF and PcF-like homologues more precisely and at an earlier stage than can be achieved by observing morphological symptoms.

*Acknowledgements.* This work was partly supported by grants MURST Cofin 2001 (S. R.) and Cofin 2000 (A. S.). We gratefully acknowledge Prof. U. Benatti and Dr G. Damonte (University of Genova) for the LC/MS mass determination. We also acknowledge the CIM, University of Parma, for the use of the CD equipment.

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