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Elicitin 172 from an isolate of *Phytophthora nicotianae* pathogenic to tomato☆

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Abstract

Elicitin 172, an acid protein with elicitor activity, has been isolated in true form from culture filtrates of *Phytophthora nicotianae*, the causal agent of crown and root rot of tomato (*Lycopersicon esculentum*). The M_r (10,349 ± 1) of the purified protein, determined by ES–MS, is identical to that calculated for parasiticein using the mean isotopic composition and assuming the occurrence of three disulfide bridges. The primary structure of elicitin 172, determined using also MALDI–MS experiments, shows complete identity with parasiticein, with elicitin 310 and a cloned elicitin gene from *P. parasitica* (= *P. nicotianae*), confirming conservation of the elicitin sequence within a single species. The protein induces necrosis (hypersensitive reaction) on tobacco, but no symptoms on tomato, when applied on the leaves. Tomato pretreated with elicitin 172 was affected by *P. nicotianae*, as well as by the phytotoxic aggregates, naturally occurring with the elicitin in the non permeated dialysis fraction of culture filtrates. Finally, the elicitin induce protection of capsicum (*Capsicum annuum)* and vegetable marrow (*Cucurbita pepo*) from *P. capsici*.

Keywords: *Lycopersicon esculentum*; Soelanaceae; Tomato; *Phytophthora nicotianae*; Fungus; Elicitins; Amino acid sequence; Parasiticein

1. Introduction

The interaction between a plant and an incompatible pathogen is often associated with defense responses in plant tissues (Dixon & Lamb, 1990). The incompatibility is characterized by a hypersensitive reaction (HR), which involves the rapid death of the infected cell and the elaboration of inducible defense. Many elicitors of necrosis have been described and are considered to be responsible for the induction of hypersensitivity (Ebel, 1986).

Necrosis inducing factors have been found in the fungal culture filtrates and have been purified for *Phytophthora cryptogea*, *P. cinnamomi*, *P. capsici* (Billard et al., 1988), *P. drechsleri* (Huet, Nespoulous, & Pernollet, 1992), *P. parasitica* (Nespoulous, Huet, & Pernollet, 1992), which has been reclassified as *P. nicotianae* (Ho & Jong, 1989), and *P. cactorum* (Dubery, Meyer, & Bothma, 1994). These factors have been identified as 10 kDa nonglycosylated proteins.

P. nicotianae has a very broad host range (Hall, 1993); it is pathogenic to woody plants such as citrus and many herbaceous and ornamental plants. The pathogen is very common and wide spread and causes rotting of the crown and roots of tomato plants. In some areas, abroad, it is considered the most aggressive disease of this crop (Satour & Butler, 1967). This paper reports the chemical and biological characterization of a protein with elicitin action, purified from the culture filtrates of a strain of *P. nicotianae* (Ph. 172), isolated from tomato (*Lycopersicon esculentum)*, but not virulent on tobacco plants (*Nicotiana tabacum)*. Moreover, its complete amino acid sequence,

☆ This paper is dedicated to the memory of Professor G. Randazzo.

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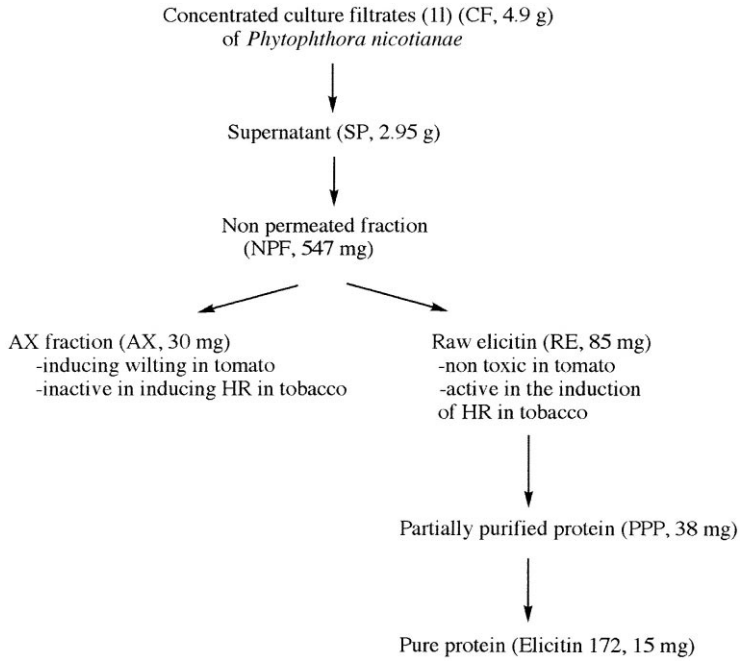

Scheme 1.

determined also by MALDI–MS experiments, is reported and shown to be identical to that of parasiticein (Nespoulous et al., 1992), elicitin 310 (Mouton-Perronnet et al., 1995) and a cloned elicitin gene from *P. parasitica* (Kamoun, Young, Glascock, & Tyler, 1993). Finally its role as elicitor is discussed.

2. Results and discussion

The purification of elicitin 172 was performed by a five step procedure (Scheme 1).

The acetone supernatant of culture filtrates from *P. nicotianae* was dialyzed and the corresponding non permeated fraction (NPF) was gel-filtered through a column of Sephadex G-50 fine, producing two very active biological fractions (AX and RE, Fig. 1a). The phytotoxic AX fraction, which caused complete wilting on tomato, but was unable to induce a hypersensitivity response on tobacco, contained aggregates of a phytotoxic peptide, named phytophorin, which has recently been characterized (Capasso, Cristinzio, Evidente, Visca, & Parente, 1997). The RE (raw elicitin) fraction was not toxic to tomato but actively induced HR in tobacco plants. The RE fraction was rechromatographed on a column of Sephadex G-50 fine and the partially pure protein (PPP, Fig. 1b) was finally purified by low pressure chromatography through a reverse phase column (Lichroprep RP-18), giving rise to a very sharp and intense peak (Fig. 1c). The protein (PP, 15 mg), named elicitin 172, was obtained in high purity grade as shown by native or SDS–PAGE.

The purification procedure, which combines gel filtration chromatography with reverse phase-low pressure chromatography, is reported for the first time for the purification of an elicitin from *Phytophthora* species. It allowed the phytotoxic aggregates containing phytophorin (Capasso et al., 1997) to be separated from elicitin 172.

After staining the tricine-SDS–PAGE electrophoregram with silver nitrate or with Coomassie blue, a single band appeared. Its apparent M_r was 10,000. In addition, the protein had an isoelectric point below pH 4, as indicated by IEF; its acidic nature is confirmed by the alkaline PAGE electrophoregram. The protein was eluted from a Sephadex G-50 fine column with urea 6 M as a single peak with an elution volume very close to that obtained when the protein was eluted with ultrapure water (Fig. 1b). The protein appeared as a single band with M_r 10 kDa on SDS–PAGE, even after heating in presence of 2-mercaptoethanol. These results, taken together, indicate that the protein has a monomeric nature. The protein, analyzed by ES–MS, exhibited the characteristic bell-shaped distribution of multiple charged ions from which a M_r of $10{,}349 \pm 1$ was measured.

The experimental amino acid composition (Ala8, Asx8, Cys6, Glx6, Gly3, Ile3, Leu10, Lys3, Met3, Phe3, Pro4, Ser12, Thr19, Tyr4, Val6) of the purified elicitin 172 accounts for a total of 98 amino acid residues and a M_r of 10,350, considering the presence of three disulfide bridges. Both values are identical to those reported for parasiticein (Nespoulous et al., 1992). In addition, like parasiticein, elicitin 172 does not contain hystidyl, arginyl or tryptophanyl residues.

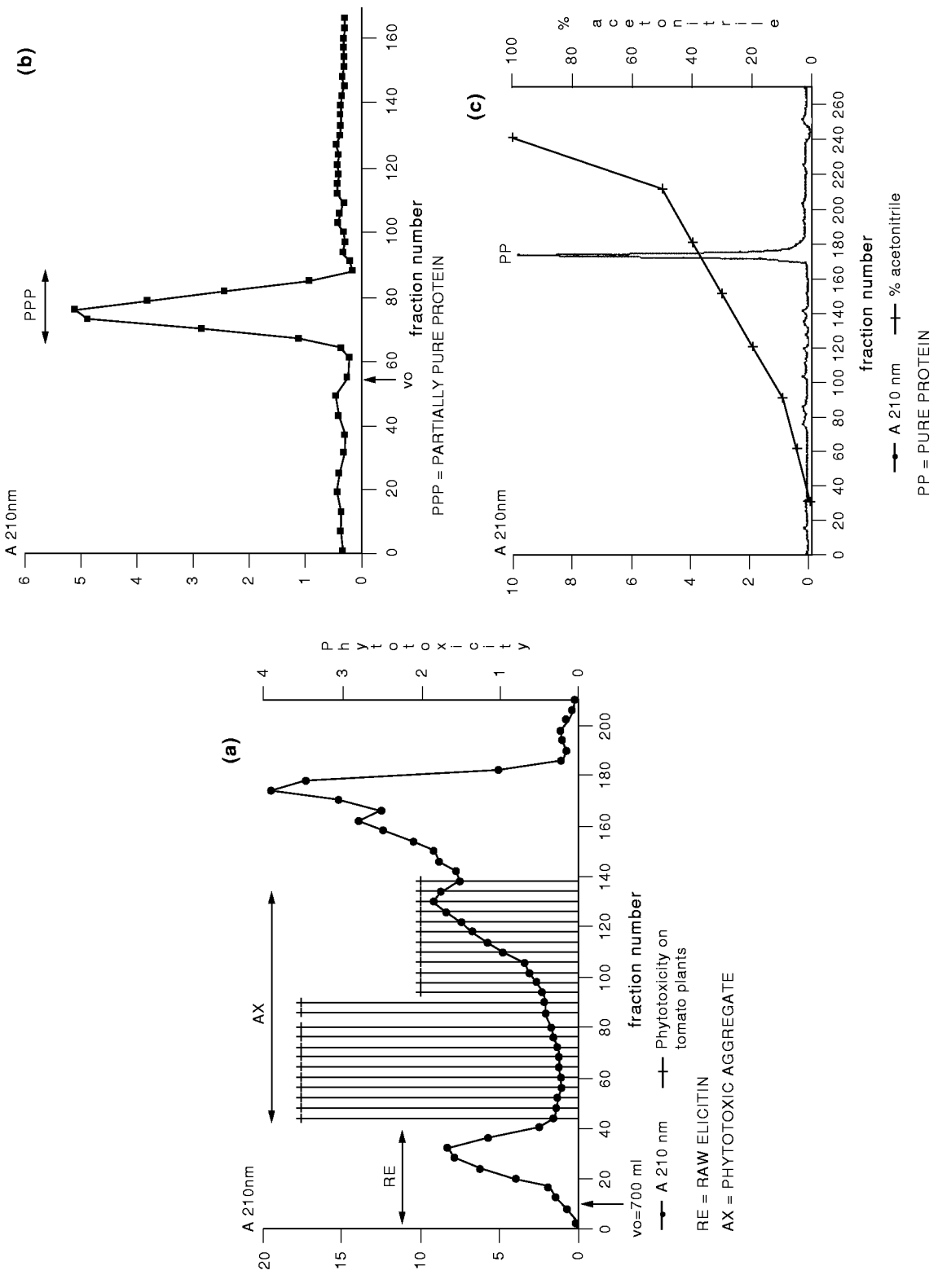

Fig. 1. (a) Elution profile from the Sephadex G-50 fine column of nonpermeated fraction coming from dialysis of supernatant, obtained from acetone precipitation of culture filtrates of *P. nicotianae*. The sample (547 mg) was applied on a column (170 × 4.2 cm) of Sephadex G-50 fine eluted with ultrapure water at a flow rate of 10 ml/30 min. RE Fraction induced hypersensitive reaction on tobacco plants. (b) Elution profile from Sephadex G-50 fine of the proteic fraction 1–43 coming out of gel filtration. Column: 72 × 3.2 cm. The eluent was ultrapure water, the flow 3 ml/11 min. (c) The insert shows the rechromatography of the sample PPP by RP–LPLC on a C-18 column (31 × 2.5 cm) using as eluent H_2O/MeCN mixed in stepwise under low pressure (3 bar). Flow rate was 15 ml/min, fractions of 5 ml.

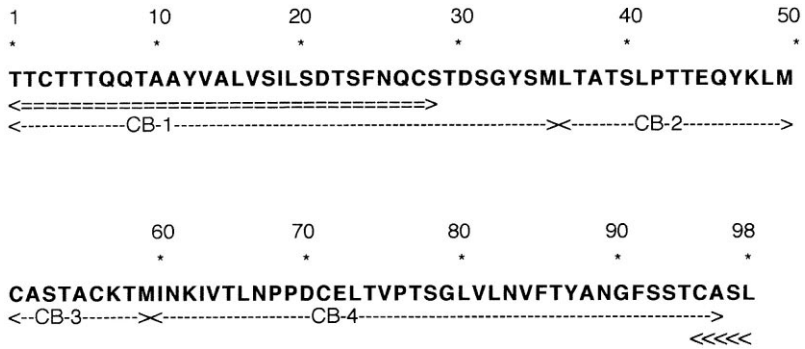

Fig. 2. Complete amino acid sequence of elicitin 172. Automated Edman degradation (⇔, ↔) and CPase A digestion (< < < < <), followed by MALDI–MS were employed for sequence determination. CB-peptides are numbered according to their position in the polypeptide chain. Alignment of CB peptides 2 and 3 was obtained by homology to other elicitins. CB means cyanogen bromide.

Therefore, here we confirm that threonine, serine and leucine are the most abundant amino acid residues; that the acidic nature, indicated by the experimental pI value, is consistent with the presence of eight Asx and six Glx and of only three lysyl residues and the absence of hystidyl and arginyl residues and that the UV absorbance with a maximum at 277 nm (not reported) is in accordance with the presence of phenylalanyl and tyrosinyl residues and the absence of tryptophan.

Automated Edman degradation both on the native and *S*-pyridylethylated protein, allowed the N-terminal sequence of elicitin 172 to be identified up to residue 28. Furthermore, the Edman degradation analysis detected at least one isoform in the apparently homogeneous elicitin 172. This contained an alanyl residue in position 4, with an occurrence of about 15%. The rest of the parasiticein sequence came from the CB-peptides (Fig. 2). Native elicitin 172 was treated with CNBr and then subjected to RP–HPLC chromatography. This step furnished peptide CB-2 (residues 36–50), which was completely sequenced by Edman degradation. Peptides CB-1 (1–35), CB-3 (51–59) and CB-4 (60–98) were eluted as a single peak and were separated by *S*-pyridylethylation and RP–HPLC chromatography. CB-1 and CB-3 were completely sequenced by Edman degradation, while CB-4 was sequenced up to residue 96. The last two residues were obtained by combining CPase A digestion with MALDI–MS analysis. In particular, aliquots of CB-4 peptide incubated with the enzyme were analyzed at five minute intervals and the amino acid residues removed in sequence from the C-terminal peptide were identified by the mass change relative to the mass (4311.9) of CB-4 peptide, as shown in Table 1. Therefore the C-terminal sequence was determined as Cys–Ala–Ser–Leu. From the alignment of the CB-2 and CB-3 peptides it was inferred from the sequence identity with elicitin 310 (Mouton-Perronnet et al., 1995).

As in parasiticein, the six cysteine residues are located in the elicitin 172 in positions 3, 27, 51, 56, 71 and 95 and have disulfide bridges. This conclusion is based on the cyanogen bromide reaction, as reported above, and because of the reaction with Nbs_2 (5,5′-ditiobis-2-nitrobenzoic acid) (Parente, Merrifield, Geraci, & D'Alessio, 1985) which showed no free sulphydryl groups. Moreover, the occurrence of three disulfide bridges is in agreement with a M_r of 10,350, found for the elicitin 172 by mass spectrometry (see above). All these data suggest that elicitin 172 is identical to parasiticein. We believe that the sequence reported for parasiticein by Nespoulous et al. (1992), with Ser in position 65 is a typographical error, as the Authors report an M_r of 10,350 and not 10,336 for a seryl residue. This is confirmed by the mass value from MALDI–MS for the intact CB-4 (4311.9 with two pyridylethylated cysteinyl residues) which is consistent with the occurrence of a Thr residue in position 65. Anyhow, the complete sequence of elicitin 172 was identical to that determined by Kamoun et al. for a cloned elicitin gene from *P. parasitica* (Kamoun et al., 1993) and to elicitin 310 (Mouton-Perronnet et al., 1995). It is interesting to recall that parasiticein is obtained from a carnation isolate of *P. nicotianae* and that elicitin 310 is produced by an Australian tobacco isolate of *P. nicotianae* (Mouton-Perronnet et al., 1995).

The conservation of the same primary structure in a single species of *P. nicotianae*, as indicated by parasiticein, elicitin 310 (Mouton-Perronnet et al., 1995) and

Table 1
Time course digestion of CB-4 peptide with Cpase A[a]

t (min)	M_r (uma)	ΔM_r	Removed residue
0	4311.9	–	–
5	4198.9	113.0	Leu/Ile
10	4111.8	87.1	Ser
15	4040.4	71.4	Ala
20	3832.3	208.1	Cys (pyridylethyl)

[a]Experimental conditions were as reported in the text.

by the cloned elictin gene from *P. parasitica* (Kamoun et al., 1993), is confirmed by elicitin 172. The identity of elicitin 172 with parasiticein and elicitin 310 was also confirmed by the hypersensitive reaction which it induced on tobacco. In fact from 0.2 to 20 μM, elicitin 172 induced necrosis on tobacco, when applied on the leaves; when tobacco leaves were immersed in a soln of elicitin 172 at 1 μM, little necrotic spots appeared on tobacco leaves; at 10 μM, necrosis was observed on entire leaves. In the same experiments performed at the same concentrations on tomato with elicitin 172, no symptoms were observed. The inactivity of the protein on tomato was confirmed in the exogenic resistance induction experiments carried out using elicitin 172 at concentrations ranging from 0.1 to 5 μM; the tomato plants, later inoculated with *P. nicotianae* were all affected by the fungus at 4000 zoospores/ml. In addition, the phytotoxic aggregates, containing phytophorin (Capasso et al., 1997) conserve their activity on tomato in the presence also of elicitin 172, as shown by the NPF fraction described above and in Fig. 1.

By performing the exogenic resistance induction experiments on capsicum and vegetable marrow plants, pretreated with the elicitin at 5 μM, inoculated with *P. capsici*, the plants remained 60 and 50% unaffected, respectively, in addition the tissues of vegetable marrow, pretreated with the elicitin at 5 and 10 μM, remained only partially damaged: 12 against 20 mm of the control. Therefore elicitin 172 induces a significant protection in different plant–pathogen systems like *P. capsici–Cucurbita pepo* and *P. capsici–Capsicum annuum*, suggesting the possible employment of this substance in an integrated pest management program.

3. Experimental

3.1. General

Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

3.2. Production of culture filtrates

A pathogenic strain of *P. nicotianae* was isolated from tomato plants in Paestum (SA), deposited in the culture collection at the Istituto di Patologia Vegetale, Università di Napoli 'Federico II' and numbered Ph. 172. The stock culture was maintained on V-8 agar petridishes. Liquid cultures were obtained by growing the fungus in flasks containing ASYT medium composed of: 1 g L-asparagine, 10 g sucrose, 5 g yeast extract, 1 mg thiamine, 0.5 g $KH_2PO_4 \cdot 7\ H_2O$ and 1 l H_2O. The flasks were incubated with stirring for 12 days at 26°C. Then, the culture fluid was strained through cheese cloth and filter paper, sterilized by passage through a 0.22 μm filter and stored at −20°C.

3.3. Purification of elicitin 172

Culture filtrates (1 l, 4.9 g) were concd by evapn under vacuum to 500 ml. This preparation was treated with 3 vol of $(Me)_2CO$ at −10°C and allowed to precipitate for 0.5 h. The acetone was removed from supernatant by evaporation under red. pres. The ppt was collected by centrifugation at 10,000 rpm for 15 min. The supernatant (2.95 g) was dialysed using tubes with a cut-off of 3.5 kDa: the nonpermeated fraction (547 mg) was lyophilized and the impure residual protein was purified using a 3-step chromatographic procedure. In the first step 547 mg of lyophilized material were applied to a Sephadex G-50 fine column (Pharmacia). Elution was carried out with ultrapure Millipore H_2O; fractions of 10 ml were collected and their *A* monitored at 210 nm. The active fractions (85 mg) were pooled according to the UV diagram (Fig. 1a), lyophilized and further chromatographed on a column of Sephadex G-50 fine. Fractions of 3 ml were obtained and their *A* monitored at 210 nm (Fig. 1b). In the third step the partially purified protein (38 mg) was finally chromatographed (15 mg each time) through a Lichroprep RP-18 column (Merck) and eluted stepwise with H_2O containing MeCN (0, 1, 5, 10, 20, 30, 40, 50 and 100% of MeCN in H_2O, 150 ml every step) at low pressure (3 bar). Fractions of 5 ml were collected and monitored as described above (Fig. 1c). The purity of elicitin 172 in every step was controlled by SDS and alkaline native PAGE.

3.4. PAGE

Polyacrylamide gel electrophoresis in Na dodecyl sulphate (SDS–PAGE) was performed on 12.5–15% polyacrylamide gels at pH 8.8 with glycine–Tris buffer according to Laemmli (1979). Values of the relative M_rs of protein bands were estimated by comparison with the following standards (Bio–Rad): phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). SDS–PAGE was performed also using Tricine–SDS or Tricine–SDS with urea 6 M, according to Schägger and von Jagow (1987). In this system values of the relative M_rs of protein bands were estimated by comparing them with the following M_r standards (Sigma): myoglobin polypeptide backbone (1–153, 16.95 kDa), myoglobin I + II (1–131, 14.44 kDa), myoglobin I + III (56–153, 10.6 kDa), myoglobin I (56–131, 8.16 kDa), myoglobin II (1–55, 6.21 kDa), glucagon (3.48 kDa), myoglobin III (132–153, 2.51 kDa). The samples employed in electrophoretic run

were denaturated with or without 5 vol% 2-mercaptoethanol and incubated for 5 min at 95°C. Isoelectrofocusing (IEF) was performed on PhastGel precast mini-gels (Pharmacia) with a pH range of 3–9. The isoelectric point was estimated with marker proteins (Pharmacia): phycocyanin (p*I* 4.65), β-lactoglobulin B (p*I* 5.10), bovine carbonic anhydrase B (p*I* 5.85), human carbonic anhydrase B (p*I* 6.55), horse myoglobin (p*I* 7.00), human hemoglobin A (p*I* 7.10), human hemoglobin C (p*I* 7.5), lentil lectins (p*I* 8.15, 8.45, 8.65), cytochrome c (p*I* 9.60). Alkaline PAGE was performed at pH 8.3 with glycine–Tris buffer according to Davis (1964). After the run, the gels were stained with either Coomassie Blue (Fazekas, Webster, & Datyner, 1963) or $AgNO_3$ (Blum, Beier, & Gross, 1987).

3.5. Cyanogen bromide cleavage

Cleavage of native elicitin 172 with CNBr was carried out in 70% TFA as already reported (Parente et al., 1993).

3.6. CPase A digestion

CNBr peptide CB-4 was dissolved in 0.02 M NH_4HCO_3 and incubated with CPase A (Sigma; final enzyme:substrate ratio of 1:50, w:w), at 37°C. Aliquots of 10 μl were removed from the incubation mixture after 0, 5, 10, 15 and 20 min and analyzed by MALDI–mass spectrometry.

3.7. Mass spectrometry

Elicitin 172 was analyzed by ES–MS using a platform single quadrupole mass spectrometer (Micromass) aliquots (10 ml ~ 1 mg) of protein soln were injected into the ion source at a flow rate of 2 ml/min. The mass spectrometer was scanned from m/z 1000 to m/z 2500 at 10 s/scan, using a cone voltage of 3.6 kV and an orifice voltage of 40 V. Timed aliquots (1 ml, about 0.5 mg) of CPase A-treated CB-4 were analyzed by MALDI–MS, using α-cyano-4-hydroxycinnamic acid (Fluka), as matrix, prepared in the following way. The sample was loaded on the target and dried. Then 1 ml of a mixture composed by 0.1% TFA in H_2O–EtOH 10 mg/ml of matrix in H_2O (l:1:1 v/v) was added. The sample was analyzed using a Voyager mass spectrometer in linear mode.

3.8. Amino acid composition

Samples of native or *S*-pyridylethylated elicitin 172 were hydrolysed at 110°C for 20 h in the presence of 0.1% phenol. Amino acid analyses were obtained with an amino acid analyzer equipped with the postcolumn, ninhydrin detection system. Chemicals and experimental were as suggested by the manufacturer.

3.9. S-pyridylethylation

Samples of native (50 μg) or CNBr-treated (25 μg) elicitin 172 were *S*-pyridylethylated as reported (Scudiero et al., 1995). At the end of the incubation time the soln was brought to 50% HCOOH and chromatographed by RP–HPLC on a Vydac C4 column, using MeCN containing 0.1% TFA as eluant. Alternatively, to prevent protein losses, upon precipitation, the modified elicitin was gel filtered on a G-25 column, in the presence of 6 M Guanidine·HCl and desalted when needed.

3.10. Amino acid sequencing

Samples of native, *S*-pyridylethylated elicitin 172 or CB- peptides were sequenced by automated Edman degradation on a pulsed phase sequencer as reported (Parente et al., 1993).

3.11. Phytotoxic activity

Phytotoxicity of the fractions achieved by successive steps of purification was assessed on tomato cotyledonary leaflets (cv. Marmande). Cotyledonary leaflets were maintained for 3 days in 200 μl of the soln to be assayed. Phytotoxic activity was scored from 0 (not toxic) to 4 (highly toxic) on the basis of leaf wilting and necrosis, according to the procedure of Graniti (1957).

3.12. Induction of hypersensitivity

Induction of hypersensitivity by pure elicitin 172 was determined by infiltrating sterile distilled H_2O solns of protein into tomato leaves (cv. Marmande) and tobacco plants (cv. Rustica). A hypersensitive response (HR) was scored when a brown necrosis occurred in the infiltrated area, 48 h after inoculation. HR was scored on the basis of necrotic areas: 0 means a necrosis of 0–5% infiltrated tissues; 1: 6–25% infiltrated tissues; 2: 26–50% infiltrated tissues; 3: 51–75% infiltrated tissues; 4: 76–100% infiltrated tissues. To test the induction of distal HR, petiole dip assays (Huet et al., 1992) were carried out on cut tomato (cv. Marmande) and tobacco (cv. Rustica) leaves. Leaf petioles were dipped into a 50 ml soln of protein. The soln was taken up after about 2 h. Then the leaves were transferred to sterile H_20. Necrotic lesions were visible after 24–48 h and ranged from minute necrotic spots to large, confluent necrotic areas.

3.13. Protection activity

Pure elicitin 172 was applied to 20 day-old tomato plants. The roots were excised and the epicotyls were dipped into elicitin 172 solns at different concens. Then the plants were inoculated with *P. nicotianae* zoospores at different concens. Elicitin 172 was applied to 20-day old capsicum excised (cv. Yolo Wonder) and vegetable marrow plants (cv. S. Pasquale). The plants were treated with different concens of elicitin 172 and then inoculated with zoospores of an isolate of *P. capsici* numbered Ph. 275 from the culture collection at the Istituto di Patologia Vegetale, Università degli Studi di Napoli 'Federico II'. Elicitin 172 was applied to 14-day old vegetable marrow plants (cv. S. Pasquale). The stem was decapitated and a 20 μl drop of elicitin 172 soln or the same vol. of sterile distilled H_2O was placed onto the fresh wound. Then the plants were inoculated with an agar plug taken from a V-8 agar plate inoculated with *P. capsici*.

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