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Characterization of elicitin-like phospholipases isolated from *Phytophthora capsici* culture filtrate

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Abstract The phytopathogenic oomycete *Phytophthora capsici* secretes in culture a phospholipase activity. Two enzyme isoforms exhibiting a high phospholipase B activity were isolated by chromatography and electrophoresis. They differ in their apparent molar masses (22 and 32 kDa). Both proteins are glycosylated and share the same N-terminal amino acid sequence up to the 39th residue with a high homology with capsicein, the *P. capsici* elicitin. Although devoid of phospholipase activity, capsicein was shown by circular dichroism to specifically interact with negatively charged phospholipids, suggesting that the membrane lipids could be a potential target for elicitins.

Key words: Circular dichroism; Elicitin; Phospholipase; Sequence; *Phytophthora*

1. Introduction

The interactions between the phytopathogenic oomycete *Phytophthora* and host plants are supposed to involve elicitins, which are 10 kDa proteins abundantly secreted into the medium. Elicitins cause not only remote leaf necrosis in infected tobacco plant, but also act as signals in the plant-pathogen interaction. They indeed generate a hypersensitive-like reaction, inducing a systemic acquired resistance, in some plants, against microbial plant pathogens [1,2], but the biological role of elicitins and their mode of action are currently puzzling [3]. A recent report demonstrated a role for an elicitin in the resistance of a *Nicotiana* species to *Phytophthora infestans* [4]. Although a succession of cellular events occurring in response to elicitins was described [3,5–7], to date, little is known concerning the molecular mechanisms involved in their activity, in spite of extensive sequence information [8–10], three dimensional (3D) determination of the β-cryptogein [11,12] and definition of necrotic and defense inducing sites in the molecule [9,10]. However, the plasmalemma was shown to be involved in the early events of signal transduction [13], suggesting that it would be the first target of elicitins in the necrotic process. However, the nature of this interaction has not yet been elucidated, although specific high-affinity binding sites for β-cryptogein, an elicitin secreted by *Phytophthora cryptogea*, were observed in tobacco plasma membranes [14].

The overall structure of β-cryptogein corresponds to a novel folding type without homology with any known protein structure [11,12]. No biological function can thus be assigned to elicitins in relation with known structural motives. Nevertheless, elicitins were recently shown to bind sterols [15] and the 3D structure of the ergosterol β-cryptogein complex was solved by X-ray crystallography [16]. The comparison of elicitins with known proteins revealed that the secondary structure and the disulfide bridge location are comparable with those of animal secretory phospholipases A_2 [17]. This structural analogy led us to examine the phospholipase activity of elicitins, which was indeed observed to be associated with α elicitin fractions [18], but not with β elicitins [19]. This observation can be interpreted by the presence, in the α elicitin fractions, of contaminating proteins bearing a phospholipase activity not separated during the α elicitin purification procedure, which is different from that of the β elicitins [20]. In this report, we describe the purification and characterization of the proteins secreted by *Phytophthora capsici* bearing a phospholipase activity in order to compare them with elicitins.

2. Materials and methods

2.1. Purification

Phospholipases were purified from culture media of *P. capsici* (strain A147) and *P. cryptogea* (strain A52). *Phytophthora* species were grown for 8 days at 27°C in a liquid medium adapted from Hall et al. [20,21] before collection. After sterilization by filtration through a 0.45 μm hydrophilic poly vinylidene difluoride (PVDF) membrane (Millipore), the culture filtrate (1.8 l) was loaded onto QAE Zeta-Prep disks (disk 60, Cuno) equilibrated with buffer A (10 mM Tris, pH 8) at a flow rate of 2.5 ml/min per disk using a peristaltic pump. The anion exchange chromatography elution was carried out with a stepwise salt gradient (0.1, 0.3 and 0.5 M NaCl in buffer A). The active fraction was desalted and concentrated by ultrafiltration using a YM3 membrane (Amicon). In a second step, it was dissolved in buffer B (1 M ammonium sulfate, 25 mM phosphate buffer, pH 7.5) and subjected to hydrophobic interaction chromatography (HIC) on a 75×7.5 mm Hema-HIC 300 column (Alltech) equilibrated with buffer B. Proteins were eluted at a flow rate of 0.5 ml/min using a decreasing ammonium sulfate gradient (1–0 M) in 25 mM phosphate buffer, pH 7.5. After desalting and concentration by ultrafiltration, the active fractions were submitted to an exclusion-diffusion chromatographic step on a 30×0.75 cm TSK G3000SW column (Chrompack) equilibrated with 25 mM phosphate buffer, pH 7.5. Elution was performed with the same buffer at a flow rate of 0.25 ml/min. All experiments were UV-monitored at 280 nm. The protein concentration of the chromatographic fractions was evaluated by Bradford determination after precipitation by 10% TCA (Pierce) or by spectrophotometric measurement at 280 nm assuming an average

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Abbreviations: $bpPLA_2$, bovine pancreatic phospholipase A_2; CD, circular dichroism; DMPC, L-α-phosphatidyl-choline di-myristoyl; DMPG, L-α-phosphatidyl-glycerol di-myristoyl; GC, gas chromatography; HIC, hydrophobic interaction chromatography; OMPC, L-α-phosphatidyl-choline β-oleoyl γ-myristoyl; POPC, L-α-phosphatidyl-choline β-palmitoyl γ-oleoyl; PVDF, poly vinylidene difluoride

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absorbance of one for a concentration of 1 mg/ml. In parallel with the phospholipase purification, the elicitin capsicein was further purified by DEAE chromatography on a 75×7.5 mm TSK DEAE-5PW column (Merck) using a gradient of NaCl (0–0.5 M) in buffer A at a flow rate of 1 ml/min, after the separation of the 0.1 M NaCl fraction by QAE chromatography. β-Cryptogein was purified as described [19,20].

2.2. Electrophoresis

Analysis of active chromatographic fractions was performed by SDS-PAGE (16%) according to the method of Schägger and von Jagow [22] with modification [23], using a Bio-Rad mini-Protean II system. After lyophilization, samples were dissolved in 15 μl of the SDS-PAGE buffer (12% glycerol, 4% SDS, 3% dithioerythritol, 50 mM Tris, pH 6.8). Gels were stained with 0.025% Serva blue G250 in 10% acetic acid.

Fractions obtained after the last chromatographic step were separated by native PAGE (12.5%) according to the Laemmli procedure [24], in which SDS was omitted. After lyophilization, samples were dissolved in 15 μl buffer composed of 20% sucrose and 62.5 mM Tris-HCl, pH 6.8. Electrophoresis was performed using a Bio-Rad mini-Protean II cell in Tris-glycine buffer for 1 h with a constant current of 40 mA. Gels were stained using 0.035% Serva blue R250 in 12% trichloracetic acid/5% ethanol, reverse-stained with zinc-imidazole [25] or stored unstained at −80°C for further investigations.

The apparent molecular masses of the active proteins were determined by submitting the native electrophoresis bands containing the active proteins to SDS-PAGE (16%), run on a 1 mm thick gel. Bands containing the proteins were first localized in the native gel, either by reverse zinc-imidazole staining or as follows. Samples were put in three contiguous lanes of the native electrophoretic gel. After electrophoresis, the middle lane was cut out and stored unstained. Both the right and left lanes were Coomassie blue-stained. Bands containing the proteins were localized in the unstained lane by putting it between the two Coomassie blue-stained lanes. The bands were then cut out with a scalpel, put into microtubes and eventually destained during 30 min in 25 mM Tris/192 mM glycine, pH 8.3, in the case of zinc staining. After 30 min incubation at 40°C in 50 μl SDS-PAGE buffer, the bands were placed in the wells of the 1 mm thick gel. The wells were filled with SDS-PAGE buffer containing 0.005% bromophenol blue. Electrophoresis was conducted as above for SDS-PAGE (16%). Gels were stained with silver nitrate (Bio-Rad kit).

2.3. Phospholipase activity assay

The phospholipase activity of the chromatographic fractions was tested by measuring the fatty acids release by a spectrophotometric CoA-coupled assay [26]. The reaction medium contained 5 mM L-α-phosphatidyl-choline di-myristoyl (DMPC) (Sigma), 10 mM Triton X-100, 10 mM EDTA in 50 mM Tris, pH 7.5. The medium was sonicated in ice for 1 min with pulsation to obtain an optically clear micelle solution. The incubation medium consisted of 45 μl reaction medium and 5 μl sample (5 μl Tris buffer as control). The assay was run for 2 h at room temperature in microtiter plates continually shaken. The amount of free fatty acid produced was quantified by the NEFA C reagent kit (Wako Chemicals) purchased from Oxoid (Dardilly, France) and according to the supplier's protocol adapted to our conditions (50 μl of reagent A and 100 μl reagent B). The optical density was measured at 550 nm in a microtiter plate reader (Titertek Multiskan Plus MK II, Flow Laboratories).

The phospholipase activity of proteins separated by native electrophoresis was directly measured in the polyacrylamide gel with a similar method. Bands were localized as described above. Gel pieces were crushed in microtubes and homogenized with a plastic pestel. After addition of 40 μl 50 mM Tris, pH 7.5, a second homogenization was carried out. The enzymatic reaction was started by addition of 10 μl five times concentrated reaction medium in 50 mM Tris, pH 7.5. The microtubes were continually shaken for 4 h. After the addition of fatty acid quantification reagents, the microtubes were centrifuged for 10 min at 24 000×*g* (Universal 16R, Hettich) and 150 μl supernatant of each tube was transferred in a microtiter plate for reading at 550 nm.

2.4. Carbohydrate detection

The proteins separated on either native or denaturing unstained gels were transferred onto PVDF membranes (Problott, Applied Biosystems) using a Bio-Rad mini-Trans-Blott system in 50 mM Tris-borate pH 8.5 buffer. Electroblotting was run with a constant voltage of 80 V for 1 h. Detection of carbohydrate moieties was carried out using the ECL glycoprotein detection system (Amersham Life Science). Chemiluminescence was detected on Kodak BioMax Light films.

2.5. Protein sequencing

The proteins separated by electrophoresis were transferred onto PVDF membranes as previously described, except the constant voltage (24 V for 1 h and 60 V for 3 h). Membranes were stained a few seconds with 0.1% Serva blue R250 in 0.5% acetic acid/40% methanol and destained with 50% ethanol. Automated Edman degradation was performed using a Perkin-Elmer Procise 494-610A sequencer with the reagents and methods of the manufacturer. In native electrophoresis, cysteines were neither reducted nor alkylated and thus, could not be identified by the sequencing protocol used. After SDS-PAGE, cysteines were identified as Cys-*S*-β-propionamide [27].

2.6. Identification of the fatty acids released by phospholipases

The hydrolytic type of the phospholipase activity was determined using specific phospholipids bearing two fatty acids of different length, L-α-phosphatidyl-choline β-palmitoyl γ-oleoyl (POPC) and L-α-phosphatidyl-choline β-oleoyl γ-myristoyl (OMPC) (Sigma). L-α lyso phosphatidyl choline myristoyl was likewise used as specific substrate for phospholipase A_1 or B. Reaction media were as above except the addition of 2.5 μl of 100 mM heptadecanoic acid (in 100% ethanol) as internal standard. The reaction proceeded at room temperature for 2 h in a glass vessel closed by a teflon septum and flushed with nitrogen. It was stopped by the addition of 10 μl of 1 M citric acid and cooling at −80°C.

After addition of 2 ml H_2O, the free fatty acids present in the medium were extracted two times with 3 ml chloroform. After evaporation of the chloroform phase, samples were dissolved in 20 μl cyclohexane and pre-purified on a 15×0.46 cm Supelcosil LC-Si column (Supelco) equilibrated with a mixture of isopropanol/cyclohexane (1/99, v/v). The components present in the medium were eluted at a flow rate of 1 ml/min using an increasing gradient of isopropanol and water in cyclohexane (final concentration 19/1/80, v/v/v). The fraction corresponding to free fatty acids eluted in the void volume (3 min) and was dried. Elution was monitored using the absorbance at 215 nm.

The free fatty acids were then analyzed by gas chromatography (GC) after methylation with 3 ml BF3 (14% in methanol) for 35 min. GC analysis was performed using a GC 8000 Series 8180 apparatus (Fisons Instruments, Thermoquest, France) equipped with an on-column injector and a FID detector set at 300°C. Aliquots were injected on a DB-1 column 30 m×0.32 mm, i.d. 0.25 μm (J and W, Interchrom, France) with a 10 m×0.53 mm desactived precolumn (Interchrom, France). Helium was the carrier gas (8.7 ml/min) and the temperature gradient was from 60°C to 200°C in 7 min, a step at 200°C for 5 min, from 200°C to 295°C in 3 min and a step at 295°C for 10 min. The apparatus was calibrated using myristic, palmitic, heptadecanoic and oleic acid methyl esters. Samples and blanks were analyzed one after the other.

2.7. Necrotic activity assays

A 10 μl aliquot of capsicein in pure water and of bovine pancreatic phospholipase A_2 (bpPLA_2) in circa 10 mM Tris-HCl pH 7.5 were infiltrated into the leaf parenchyma of 60 days old tobacco plants (Xanthi) as previously described [9]. A similar amount of each protein (from 0.01 to 1 nmol) was infiltrated. The plants were placed under controlled conditions (25°C, 18 h of light) and scored for necrotic lesions 18–96 h after inoculation. Necrotic spots were observed after 24 h and did not extend thereafter. Photographs were taken after 3 days. Water and 10 mM Tris-HCl pH 7.5 buffer were used as controls.

2.8. Circular dichroism (CD)

CD spectra were obtained and analyzed as previously described [28]. Cryptogein and capsicein concentrations were determined using UV spectroscopy using a molar ε of 8,306 and 8,118 M^{-1}/cm at 277 nm, respectively, calculated from the amino acid content of the mature processed molecules [29]. Stock solutions of phospholipid (500 μM in water) were sonicated with pulsation during 1 or 2 min in ice in order to obtain an optically clear suspension of small unilamellar vesicles.

3. Results

3.1. Purification of phospholipasic proteins

Proteins bearing a phospholipase activity were purified from *P. capsici* culture medium by three successive chromatographic steps followed by native electrophoresis. After filtration, the culture filtrate of a 8 day old culture of *P. capsici* was fractionated by anion exchange chromatography. The phospholipase activity was found exclusively bound to the disk, like capsicein, confirming the acidic nature of both proteins. The active fraction, named *P1*, eluted at 0.3 M instead of 0.1 M NaCl for capsicein (named Cap) (Fig. 1a). No phospholipase activity was found in the capsicein peak. Similar results were obtained with a *P. cryptogea* culture filtrate, except that the elicitin β-cryptogein did not bind to the disk, because of its basic nature, and that the phospholipase active peak eluted between 0.3 and 0.5 M NaCl (data not shown). After this first step of purification, the active fraction corresponded to about 27 and 12% of the total proteins present in the culture medium of *P. capsici* and *P. cryptogea*, respectively. The purification was continued only with fractions obtained from *P. capsici*.

The active fraction from *P. capsici* was desalted by ultrafiltration before being subjected to HIC. After application of a decreasing salt gradient, the phospholipase activity was observed in three fractions eluting at 0.63, 0.47 and 0.18 M ammonium sulfate (Fig. 1b). The last HIC fraction, named *P2*, was the most active and abundant one (90% of the total activity). Each fraction was desalted and subjected to gel filtration. The activity of the two first HIC fractions eluted in each case as three new active peaks (not shown), whereas the chromatography of the last HIC fraction, *P2*, led to a single active peak (*P3*), which showed a slight shift with regard to the UV peak (Fig. 1c). Further investigation was focused on this latter fraction. At this stage of purification, the proteins bearing a phospholipase activity (1.15 μmol/min/mg protein) corresponded to 0.2% of all the proteins secreted by *P. capsici*.

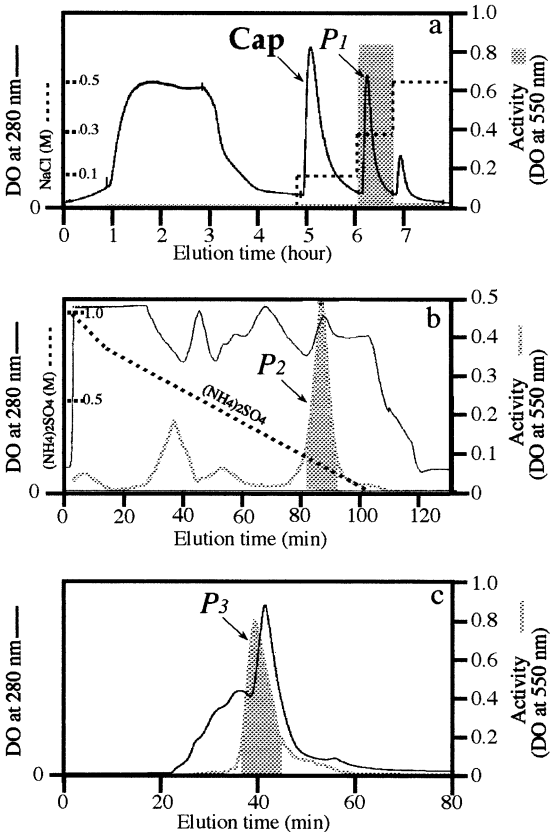

Fig. 1. Three-step chromatographic purification of phospholipase activity from *P. capsici* culture medium. a: Anionic chromatography, 1.8 l culture medium (about 200 mg protein) loaded on QAE disks. b: HIC, 20 mg of protein loaded on a HIC column. c: Exclusion-diffusion chromatography, 1.3 mg protein loaded on a TSK column. Elution was followed at 280 nm, phospholipase activity was measured by a colorimetric detection at 550 nm. The activity in (a) was measured on the whole fractions of each step of the NaCl gradient. Active fractions at each chromatography are marked *P1–P3* and the corresponding peaks shadowed. The capsicein peak is indicated by Cap.

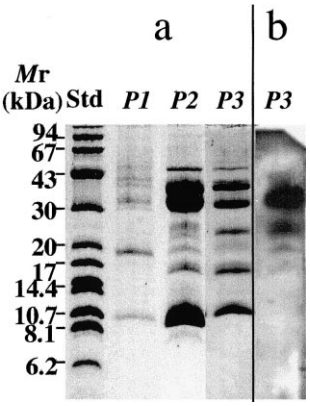

Fig. 2. Electrophoresis of the active chromatographic fractions. a: SDS-PAGE (10 μg proteins loaded onto the gel), proteins were stained with Coomassie blue. b: After SDS-PAGE, electroblot of fraction *P3* onto a PVDF membrane and detection of oligosaccharide moieties by chemiluminescence (2 μg protein loaded onto the gel).

After each chromatographic step, the active fractions were checked by SDS-PAGE (Fig. 2a). Analysis of fraction *P2* revealed at least three major bands of molar masses around 10, 32 and 38 kDa, which were still observed after the exclusion-diffusion chromatography (*P3*). Two minor bands (molar mass around 22 and 16 kDa) were proportionally more abundant at this final step. Research of carbohydrates revealed (Fig. 2b) that all the proteins present in fraction *P3* were

Table 1
Comparison of the N-terminal sequences from:

	name bands	name sequences	amount (pmol)	sequences 1 5 10 15 20
a	10kD	Cap	48	**ATCTTTQQTAAYVALVSILS** *T*
		PL	4	EAC*SA***TQQ***AS***AY***T*SMVGLL**Q**
	22kD	PL	62	EAC*SA***TQQ***AS***AY***T*SMVGLL**Q**
	32kD	PL	37	EAC*SA***TQQ***AS***AY***T*SMVGLL**Q**
b				1 5 10 15 20 25 30 35 39
	B1	Cap	30	**AT-TTTQQTAAYVALVSILSDSSFNQ-ATDXGYXMLXAT** *T*
		PL	24	EA-*SA***TQQ***AS***AY***T*SMVGLL**Q**G*TALTX*-**AS**DXG**Y**N**ML**Y**AT** *A LG TA Q*
	B2	Cap	6	**AX-TTTQQXAAY**
		PL	46	EA-*SA***TQQ***AS***AY***T*SMVGLL**Q**G*TALTT*-**AS**D**S**G**Y**N**ML**Y**AT**

a: 10, 22 and 32 kDa bands from SDS-PAGE (see Fig. 2a).
b: Active bands *B1* and *B2* from native electrophoresis (see Fig. 3). Bold characters indicate the residues identical to those of the capsicein sequence, italics character the similar residues. Dashes indicate potential cysteyl residues (not identified) according to the capsicein sequence, X indicates unidentified residues.

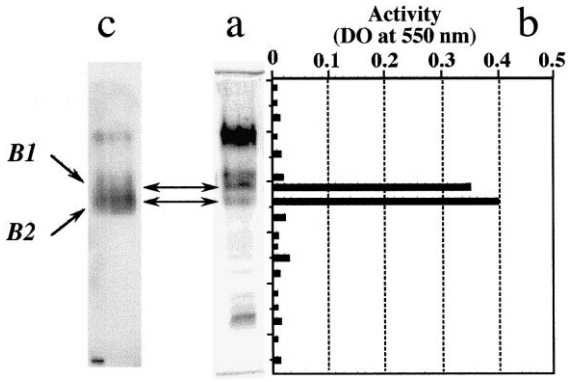

Fig. 3. Phospholipase activity of the *P3* fraction. a: Native electrophoresis (10 μg proteins loaded onto the gel), proteins were stained with Coomassie blue. b: Phospholipase activity measured by a colorimetric detection at 550 nm, along the native gel lane (10 μg protein loaded onto the gel). c: After native PAGE, electroblot onto a PVDF membrane and detection of oligosaccharide moieties by chemiluminescence (2 μg protein loaded onto the gel). Arrows indicate both active bands *B1* and *B2*.

more or less glycosylated, with the exception of the proteins of 10 and 38 kDa.

In order to locate and identify the proteins bearing the phospholipase activity, *P3* components were separated by native electrophoresis, revealing five distinct bands (Fig. 3a). The first and slowest one was the most abundant, whereas the fastest one was hardly detectable with Coomassie blue staining. The three others had a similar migration behavior. The direct activity determination in the polyacrylamide gel (Fig. 3b) showed that the phospholipase activity was located in two distinct and neighboring bands, called *B1* and *B2*, and no activity was found elsewhere along the lane. The active bands *B1* and, more strongly, *B2* were also found to be glycosylated in addition to the slowest band of the fraction (Fig. 3c).

3.2. Enzymatic characterization of the proteins found in fraction P3

The phospholipase activity present in the chromatographic and electrophoretic fractions was measured by a method that does not reveal the specificity of the hydrolytic site of the enzyme. This information was later obtained by the use of specific phospholipids bearing two different fatty acids as substrates. Fig. 4 shows a GC analysis of the reaction products after digestion by the purified fraction *P3* of the phospholipid POPC, which is composed of palmitic acid (C16:0) at the sn-2 position and oleic acid (C18:1) at the sn-1 position. The *P3* sample GC chromatogram is overlaid with a blank obtained in absence of fraction *P3*. The control chromatogram shows a slight spontaneous degradation of the phospholipid likewise detected with the colorimetric assay. Compared with the heptadecanoic acid (C17:0), used as a standard at the same concentration (5 mM) in *P3* and control samples, both C16:0 and C18:1 peaks significantly increased in the *P3* sample. The palmitic acid at the sn-2 position in the phospholipid appeared to be the fatty acid preferentially cleaved by the enzyme. Using another phospholipid as substrate, OMPC, a comparable result was obtained, i.e. the cleavage of both fatty acids with a clear preference for oleic acid at the sn-2 position (not shown). This ability to independently hydrolyze one of each ester bonds was confirmed by using a lyso phosphatidyl choline as substrate and by measuring the release of the free

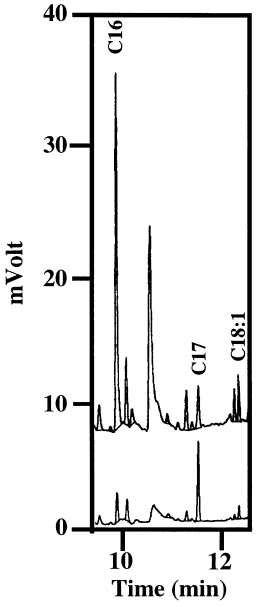

Fig. 4. GC of the products of POPC hydrolysis by the active fraction *P3* (upper curve), compared with spontaneous degradation obtained in a blank without active fraction (lower curve). The peaks corresponding to the released (C16 and C18:1) or control (C17) fatty acids are shown.

fatty acid by either colorimetric or GC methods (not shown). In addition, it is noteworthy that all the phospholipase assays were performed in absence of calcium.

3.3. Structural characterization of the proteins found in fraction P3

Both the active gel slices *B1* and *B2* were submitted to electrophoresis under denaturing conditions. Each band clearly comprised two components (Fig. 5). One is common to *B1* and *B2* with the same molecular weight as capsicein (10 kDa). The second protein is about 32 kDa in *B1* and 22 kDa in *B2*. The sequences of the 10, 22 and 32 kDa bands were determined up to the 20th residue after electroblotting of fraction *P3* from the SDS electrophoretic gel (Fig. 2a). Table 1a shows that only two types of sequences were observed, one common to the three bands and the other specific to the 10

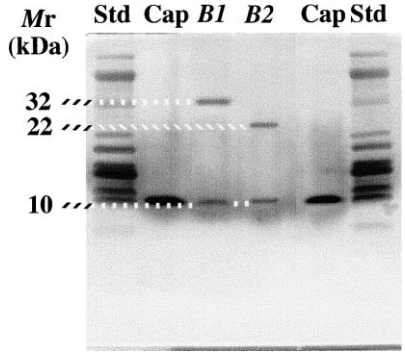

Fig. 5. SDS-PAGE of the active bands *B1* and *B2*. Gel slices containing phospholipase and capsicein (lane Cap) were put in their respective wells of the 1 mm thick gel. Std = molecular weight standard, loaded as solution. The gel was silver-stained. Molar masses of separated proteins are indicated.

kDa band. The latter (named Cap) exactly corresponded to that of capsicein [1] and to an isoform with Thr at the N-terminus instead of Ala. The other sequence, named PL, was found in both the 22 and 32 kDa bands and also in low amounts (10%) in the 10 kDa band.

The N-terminal amino acid sequences of the proteins present in the *B1* and *B2* active bands of the native electrophoresis were obtained up to the 39th residue after electrotransfer onto PVDF membranes. Table 1b shows that both *B1* and *B2* are composed of the same two main sequences, Cap and PL, which were observed in the SDS-PAGE bands. The N-terminal sequence PL is a novel one according to the protein databases (using the program BLAST 2.0 on the GenBank CDS translations, PDB, SwissProt, PIR and PRF databases). It only matched with capsicein, showing, on nearly half of the elicitin sequence, a strong identity (46%) and a close to 80% homology according to the PAM matrix and with other known elicitins [8,9]. Both sequences are in equivalent amounts in *B1* whereas the PL sequence is much more abundant in *B2*. In contrast to capsicein, which presents only one variation at the N-terminus, the PL sequence exhibited some variability at six sites among the first 39 residues in the *B1* band. This variability was not observed in the *B2* band and likely corresponds to a contaminant sequence very close to that of PL.

3.4. Necrotic activity of bovine pancreatic PLA_2

The very low amounts obtained did not allow to test the necrotic activity of the *Phytophthora* phospholipases. We nevertheless compared the necrotic activity of capsicein to that of bovine pancreatic PLA_2 by infiltration into tobacco leaf parenchyma. Tobacco did react to $bpPLA_2$ by developing a necrotic lesion, characterized by a rapid collapse of the infiltrated leaf tissues that then turned light brown and became papyraceous after 2–3 days (Fig. 6). The tested plants were responsive to both capsicein and $bpPLA_2$ at doses around 10 pmol, while water and the Tris buffer, used to solubilize capsicein and $bpPLA_2$, respectively, only gave a faint reaction restricted to the region wounded by the syringe. The $bpPLA_2$ symptoms closely mimicked the development of the hypersensitive-like reaction induced in tobacco by capsicein, in terms of dose-response, kinetics and appearance of the induced lesions (not shown).

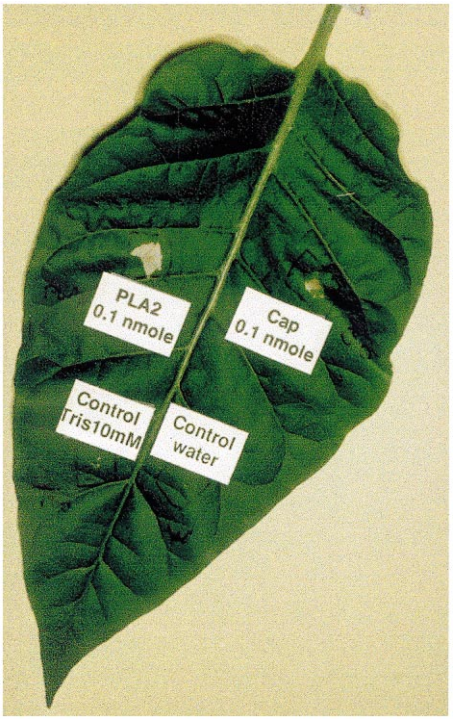

Fig. 6. Necrotic activity of bovine pancreatic phospholipase A_2 and capsicein to tobacco leaves. Infiltration of 0.1 nmol capsicein (Cap) and bovine pancreatic phospholipase A_2 ($bpPLA_2$) into tobacco leaf parenchyma. Controls: water and 10 mM Tris-HCl pH 7.5 buffer (Tris 10 mM). Photograph taken after 3 days.

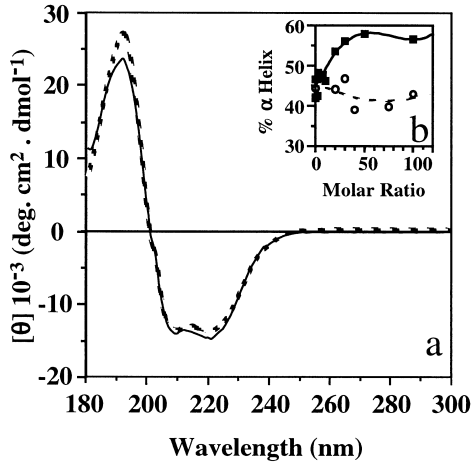

Fig. 7. a: Far UV CD spectra of capsicein (4 μM) in the presence (dashed line) or in the absence (continuous line) of 400 μM DMPG. Solvent: water, light path: 0.2 cm, room temperature. b: Evolution of the % of α-helix in capsicein as the function of the phospholipid/capsicein molar ratio. DMPG: ■, DMPC: ○.

3.5. Interaction between capsicein and phospholipids

The sequence relationships between elicitins and secreted *Phytophthora* phospholipases suggest that elicitins might interact with the plasmalemma phospholipids. We carried out a CD study of capsicein and β-cryptogein in the presence of the two most abundant phospholipids of the plant cell membranes, which differ by their polar head group, phosphatidyl-glycerol, negatively charged, and phosphatidyl-choline, which is neutral. Fig. 7a shows that the shape of the CD spectrum of capsicein changed in the presence of L-α-phosphatidyl-glycerol di-myristoyl (DMPG) by an ellipticity variation at 192 and 222 nm. Opposite to DMPG, no significant structural modification was observed in the presence of DPMC, which accounted as a negative control. In the presence of DMPG, the modification mainly consisted in an α-helix increase from 45 to 55% at a phospholipid/capsicein molar ratio of 50 (Fig. 7b), besides a slight decrease of both β-turn and -coil (not shown). Such an increase was also observed at a low SDS concentration (3 mM), which has a negatively charged polar head group such as DMPG (not shown). Similar results were obtained with β-cryptogein, which underwent a similar α-helix increase under the same conditions (not shown).

4. Discussion

P. capsici and *P. cryptogea* secrete phospholipases into their

culture medium which were isolated from *P. capsici* and characterized. A phospholipase activity was previously observed in the culture medium of *P. infestans* [30] and studied in other plant pathogens [31–33] or in other oomycetes [34]. However, to date, these *Phytophthora* enzymes were not characterized, whereas such enzymes have been extensively studied in animal pathogenic fungi [35–39]. In addition to other phospholipases in very low amounts, two major isoforms were characterized. These phospholipases of 22 and 32 kDa (the former more abundant than the latter) were shown to be glycoproteins exhibiting sequence homology solely with capsicein and other elicitins, but not with any other protein in the databanks. Although the N-terminal sequences of both phospholipases were found to be identical, they clearly differ in their apparent molar mass resulting from variations in either the sequence length or carbohydrate content. They exhibited a high activity (approximately 1 μmol/min/mg protein at pH 7.5), which explains that these enzymes were not observed as α elicitin contaminants in previous purifications due to their low amount and their close relationships with α elicitins [18]. The presence of a capsicein-like protein contaminating the PL phospholipases is puzzling since capsicein was clearly separated from the PL phospholipases at the first chromatographic step. Nevertheless, a capsicein contamination cannot be excluded because it is 1000-fold more abundant than phospholipases in the filtrate, but the presence of a novel capsicein isoform could also explain this observation. The hypothesis that the characterized molecules are not phospholipases, but contaminants that hinder the real phospholipasic molecules, cannot be totally excluded. Nevertheless, the very low abundance of the characterized molecules (less than 0.2% of the secreted proteins) compared to the high specific phospholipase activity hints that such a conclusion would be very unlikely.

The purified enzymes were shown to cleave both phospholipid ester bonds with a clear prevalence for that at the sn-2 position. Such an activity is consistent with a B type phospholipase, which is usually defined as an association of phospholipase A_2 with lysophospholipase activities. The enzymes were found to be Ca^{2+}-independent, such as some phospholipases B [38,40]. Both these features are in agreement with those of the phospholipase activity previously described in *P. infestans* [30]. It cannot be excluded that the carbohydrate moiety might be involved in the enzymatic activity as already shown for other phospholipases B [40].

Elicitins may have a phospholipase origin, which could have been promoted by the existence of a gene cluster encoding the elicitins [41], since they show a high degree of sequence homology with the purified *Phytophthora* phospholipases, at least in the first 40% of the sequence. Such sequence relationships have already been observed with other *Phytophthora* proteins such as putative glycoproteins from *P. infestans* [42], but not with another *Phytophthora parasitica* var. *nicotianae* 34 kDa glycoprotein [43].

We observed that capcisein, an α elicitin, is totally devoid of phospholipase activity, as already reported for a β elicitin [19]. The structural relationships between *Phytophthora* phospholipases and elicitins can be paralleled with animal cytotoxic proteins related to phospholipases. For instance, myotoxins from snake venom are basic phospholipases A_2 with variants lacking enzymatic activity [44]. These toxins are suggested to interact with the cell membrane, involving a region of the protein distinct from the catalytic site. Comparably, the phospholipase nigexine was shown to have cytotoxic properties independent from the esterase activity [45]. The phospholipase origin of elicitins would therefore confer some affinity for lipids to them. We indeed observed that capsicein undergoes a significant conformational change when mixed with negatively charged phospholipids organized in a bilayer. Nevertheless, neutral phospholipids do not interact with elicitins and can therefore be considered as controls showing that the relationships between elicitins and lipids is subject to some specificity. These results are in agreement with the recent finding that elicitins are able to carry sterols [15]. This interaction has been explained by close inspection of the 3D structure of an ergosterol molecule encapsulated in the hydrophobic pocket of β-cryptogein, which shows that the binding of negatively charged lipids is favored by electrostatic interactions [16]. The elicitin activity could also originate in a mechanism analogous to that of a streptococcal cytolysin, streptolysin O, which provokes the membrane disruption by binding to the sterol of the membrane, therefore decreasing its rigidity [46].

The ability of bovine pancreatic PLA_2 to induce a hypersensitive-like necrotic response in tobacco suggests that the phospholipase activity of the elicitin-like phospholipases might be directly related to the jasmonic acid defense signaling pathway occurring in vivo. The PL phospholipases might then contribute to the *Phytophthora* pathogenicity through plasma membrane hydrolysis. In addition, they could contribute to the activation of the plant defense reactions through the production of jasmonate, which is initiated by linolenic acid released as a consequence of the membrane phospholipid hydrolysis [47]. Whether $bpPLA_2$ has the potency to induce the plant defense mechanisms has now to be investigated. The present results provide new hypotheses for the functional role of elicitins, whose primary target could be the membrane lipids.

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