Biosynthesis and Secretion of Cryptogein, a Protein Elicitor Secreted by Phytophthora cryptogea

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ABSTRACT
The phytopathogenic fungi Phytophthora subspecies elicit hypersensitive-like necroses on their nonhost tobacco (Nicotiana tabacum), with the exception of the tobacco pathogen Phytophthora nicotianae. In culture, these fungi—except P. nicotianae—secrete proteins, called elicitors, that cause these remote leaf necroses and are responsible for the incompatible reaction. These proteins protect tobacco against invasion by the agent of the tobacco black shank, P. nicotianae, which is unable to produce such an elicitor. Cryptogein, secreted by Phytophthora cryptogea, has been purified, sequenced, and characterized as an elicitor, a novel family of 10 kilodalton holoproteins. In the present paper, we examined the secretion and biosynthesis of this protein elicitor from P. cryptogea culture. Results showed that the secretion of cryptogein began later than its synthesis and stopped earlier, simultaneously with mycelium growth, when the nitrogen source in the culture medium was nearly exhausted. Electrophoretic patterns of total protein from mycelium extracts and N-terminal sequence analysis showed that cryptogein accumulated in the mycelium in its mature form. The comparison of the immunoselected in vitro translation products with [35S] in vivo-labeled cryptogein showed that cryptogein was synthesized as a preprotein with a signal peptide removed cotranslationally before the secretion into the culture medium. Immunoselected in vitro-synthesized products were subjected to radiosequencing to clearly determine the N-terminal position and the size (20 amino acids) of the signal peptide. Cryptogein did not undergo any other posttranslational modification.

Numerous elicitors of necrosis produced by fungi (6) or during the plant-fungus interaction (10) have been described and are considered to be responsible for the induction of the hypersensitive reaction. They serve as signals for the interaction between plant and pathogens (11). Upon inoculation, Phytophthora nicotianae, the agent of the tobacco black shank, invades tobacco stems without causing leaf necrosis at a distance from the penetration site, whereas other Phytophthora species colonize tissues to a limited extent and cause necrosis in leaves at a distance from the inoculation site. Csinos and Hendrix (9) first showed that Phytophthora cryptogea, which is unable to parasitize tobacco plants, released into the culture medium a substance causing a severe reduction in growth of tobacco. In culture, P. cryptogea and Phytophthora capsici secreted low Mr holoproteins, named cryptogein and capsicein, respectively (3, 5); P. nicotianae did not secrete such proteins. These fungal proteins have been purified (1) even in large quantities (14). They belong to a novel family of proteins called elicitors (13). When applied to tobacco plants, elicitors not only induce systemic remote leaf necrosis after migration toward the leaf tissue from the inoculation site (22), but also cause the accumulation of pathogenesis-related proteins (4) and induce protection against a subsequent inoculation with the tobacco pathogen P. nicotianae (16). Treatment of cell suspension cultures of tobacco with cryptogein elicits the production of ethylene and phytoalexin but does not affect the integrity of the plasma membrane (2). Elicitins from various fungal species exhibit different levels of biological activities (15, 16). The complete amino acid sequence of cryptogein is known (16). It is a holoprotein of 98 residues and has a Mr of 10,329.

Because they are holoproteins devoid of side chain modification, elicitors are the only fungal elicitors that can be easily produced by genetic engineering. Nevertheless, a larger proprotein, occurring as an elicitin precursor in the biosynthesis, might be necessary for the proper folding and disulfide bridge formation. Such a project requires a precise knowledge of the biosynthesis, maturation, and secretion of the elicitin. We compared here the cryptogein secreted into the culture medium with the cryptogein accumulated in the mycelium during growth of P. cryptogea. We also compared the size and the N-terminal sequence of the immunoselected in vitro translation products of RNAs extracted from the mycelium with those of the native secreted cryptogein. We showed that cryptogein is synthesized as a preprotein with a 20-residue N-terminal signal peptide that is removed cotranslationally.

MATERIALS AND METHODS

Phytophthora Culture

Phytophthora cryptogea (isolate 52) from the Phytophthora culture collection at INRA Antibes was grown on a modified Zentmyer et al. medium (23) at 26°C in the dark with Asn (2 g L−1) as the only nitrogen source. Mycelia were collected from 2 to 28 d by filtration on 0.45 μm filters (Millipore HA type), carefully washed with pure water, then frozen in liquid nitrogen and stored at −77°C in polyethylene bags until used. For studies of elicitin secretion during culture, aliquots of the filtrate were collected from the same Roux bottle during 28 d of growth, then filtered on Costar sterile filters (0.22 μm) before quantification.

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Protein Extraction

The mycelium was ground in liquid nitrogen (Ika Werk blender) and homogenized (50 mg mL⁻¹) in a pH 7.0 buffer (20 mM Tris, 6 mM urea, 5% SDS, 3% DTT). After a 1-h extraction at 20°C, the homogenate was centrifuged at 15,000g for 15 min.

Purification of Cryptogein and Preparation of Antibodies

Cryptogein was purified from culture medium by a two-step preparative chromatographic procedure as already described (14). Rabbits were injected subcutaneously with 300 μg purified cryptogein mixed 1:1 (v/v) with Freund’s complete adjuvant. After the first injection, three injections of 300 μg antigen with incomplete Freund’s adjuvant were given at 2, 5, and 9 weeks. Rabbits were bled 1 week after the last injection. The blood was kept one night at room temperature in a Petri dish, then plasma was collected and centrifuged. The serum was stored at −20°C.

Analytical RPLC of Cryptogein

Analytical RPLC¹ was performed with a Spectra Physics system composed of an 8700 XR pump, an 8750 organizer, an 8773 XR UV detector set at 215 nm, and a 4200 integrator. Elicitins were chromatographed on a 4.6 × 30 mm Aquapore C8 RP 300 column at a flow rate of 0.5 mL min⁻¹. All solvents (UV grade) were continuously degassed with helium. The elution was performed with a gradient of CH₃CN (Fisons, far UV grade) obtained with the solvents A (CH₃CN/25 mM CH₃COONH₄, pH 7.2, 5:95, v/v) and B (CH₃CN/50 mM CH₃COONH₄, pH 7.2, 50:50, v/v). The gradient increased from 5 to 18.5% CH₃CN in 10 min, from 18.5 to 36.5% in 20 min, and from 36.5 to 50% in 5 min. A 5-min wash at 50% CH₃CN was followed by a 15-min reequilibration time at 5% CH₃CN. For quantification of elicitin in culture media, the amount of elicitin was determined from the value of the peak area with reference to purified elicitin calibration.

In Vivo ³⁵S Labeling of Cryptogein

P. cryptogea was cultured in Petri dishes in the presence of [³⁵S]sulfate (ref. SJS1, batch 223 AC, 30 GBq μmol⁻¹ from Amersham) in the culture medium described above, except that half of the MgSO₄.7H₂O was replaced with MgCl₂.6H₂O. After 15 d of culture in the dark at 25°C, culture filtrates were filtered through 0.22 μm COSTAR filters. Labeled cryptogein was directly purified by analytical RPLC. In addition to detection at 215 nm, radioactivity was monitored by continuous flow detection in a Flo-One beta radioactive flow detector performed on a 20% derivation added with Luma Flow II at 0.5 mL min⁻¹ in a flow cell of 0.5 mL. Labeled cryptogein fractions were pooled and lyophilized. The specific radioactivity varied from 2 to 5 kBq μg⁻¹.

¹Abbreviations: RPLC, reversed-phase liquid chromatography; ATZ, anilino thiazolinone.

Electrophoresis

SDS-PAGE (16.6% acrylamide) with Tricine as the trailing ion was performed with the Pharmacia LMW calibration standards and the LKB 1861-101 peptide kit with reassessed Mₐ values (17). Electrophoresis was performed on a GE 4 II Pharmacia apparatus. Nonradioactive proteins were detected by staining with Coomassie brilliant blue G-250; radioactive proteins were detected on Kodak X-Omat AR film after fluorography according to Chamberlain (7).

Western Immunoblotting Analysis

Polypeptides were electrophoretically transferred from SDS-PAGE gels to a nitrocellulose membrane (Schleicher and Schuel BA 85 0.45 μm) according to Towbin et al. (19). After transfer, the antigens were detected on the membranes with the Protoblot AP (Promega) system kit according to the procedure described by the manufacturer. The immune serum was diluted 3000-fold.

RNA Extraction and In Vitro Translation

RNAs were isolated from mycelium by a small-scale procedure (20) using a guanidinium extraction buffer (8). RNAs were translated in a commercial wheat germ system (Promega) with either [³⁵S]methionine (37 TBq mmol⁻¹, Amersharm) or [³⁵S]cysteine (37 TBq mmol⁻¹, Amersharm) as the labeled amino acid. RNA templates were used in equivalent amounts (140 μg mL⁻¹). After a 60-min incubation at 25°C, protein aliquots were precipitated on small pieces of Whatman No. 3 MM chromatography paper by immersion in ice-cold 10% TCA for 20 min. The pieces of paper were boiled for 10 min in 5% TCA, dried, and counted in Lipoluma scintillator cocktail (Kontron) with a SL3000 Intertechnique counter.

Immunofinity Isolation of In Vitro-Synthesized Cryptogein

The crude, cell-free translation assays (25 μL) were incubated with immune serum (diluted 50-fold) in 200 μL 10 mM sodium borate, pH 8.0, 160 mM NaCl, 0.5% BSA, 0.5% Triton X-100, and 20 mM methionine for 1 h at 35°C and then at 4°C overnight (12). The mixture was chromatographed over a protein A-Sepharose CL4B (Pharmacia) column. After washing the column with 10 mM sodium borate, pH 8.0, 160 mM NaCl, 0.5% Triton X-100, and 20 mM methionine, the antigen-antibody complexes were eluted with the electrophoresis sample buffer (4% SDS, 50 mM Tris-HCI, pH 6.8, and 2% β-mercaptoethanol).

Protein Sequencing and Radiosequencing

Automated Edman degradation was performed with an Applied Biosystems 475A sequencer equipped with an online phenyl thiohydantoine-amino acid analyzer model 120A, with reagents and methods of the manufacturer. Native elicitin polypeptides were spotted on glass-fiber disks and analyzed by standard procedures. In vitro translation products were recovered on Problott membrane disks (Applied Biosystems) by centrifugation according to Sheer (18) and analyzed...
with an ATZ 470-L special program. The butyl chloride extracts of ATZ-amino acid were collected directly and counted in Lipoluma/butyl chloride (10:1, v/v) mixture in a SL3000 Intertechnique scintillation counter. No carrier protein was added, because samples were antibody-antigen complexes.

Amino Acid Analysis

Culture filtrates were diluted with lithium buffer, pH 2.2. Asn was determined on a Biotronik LC 5001 analyzer with lithium buffers and ninhydrin detection.

RESULTS

Kinetics of Cryptogein Secretion into the Culture Medium and Cryptogein Accumulation in the Mycelium of P. cryptogea

Figure 1 shows the secretion of cryptogein and its accumulation in the mycelium during culture. Direct analytical RPLC was performed on aliquots of the culture filtrate (Fig. 1A) and on mycelium proteins extracted with water. Cryptogein eluted at 27.5% CH3CN; the amount of elicitin was determined at each step. As shown in Figure 1B, after 2 d of culture, the amount of cryptogein increased steeply and leveled off at day 9 (50 mg L⁻¹). Mycelium dry weight leveled off also at day 9 (13 g dry weight L⁻¹ of culture medium). The ratio of secreted cryptogein to mycelium dry weight was roughly constant during mycelial growth. Only 5% of the initial amount of Asn remained in the culture medium after 8 d of culture. The amount of cryptogein accumulated in the mycelium during culture gradually increased, then leveled off at day 14. Cryptogein in the mycelium corresponded to one-third of the total cryptogein at day 2, and nearly one-tenth later.

Characterization of Cryptogein Accumulated in the P. cryptogea Mycelium

Figure 2A shows the electrophoretic patterns of total protein extracts of P. cryptogea mycelium carefully washed from culture filtrate to avoid contamination by cryptogein released by cellular lysis. From day 2 to day 28, the electrophoretic patterns were identical for bands with Mᵣ above 20,000. However, a band with a Mᵣ of 10,000 became detectable in the course of mycelium growth, although quantities of total protein loaded were equivalent.

After electrophoresis and western blotting of mycelium extract proteins, the immobilized antigens were detected by polyclonal antibodies raised against cryptogein (Fig. 2B). The immunoblot patterns showed a band corresponding to a Mᵣ of approximately 10,000, identical to that of purified cryptogein. In another experiment, this protein of Mᵣ 10,000 was isolated by RPLC from an 18-d old mycelium extract. It was identified as cryptogein by N-terminal sequencing determined up to 20 amino acids (results not shown).

Comparison of in vitro Translation Products Directed by Mycelium RNAs to in Vivo-Labeled Cryptogein

The electrophoretic profile of in vivo 35S labeled cryptogein exhibited only one radioactive band at the same position as found for unlabelled cryptogein, corresponding to an apparent Mᵣ of 10,350 (16, 17). Total RNA extracted from 4-d-old mycelium was translated in a wheat germ system in the presence of [35S]methionine. In vitro translation products were isolated by immunofinity. Figure 3 shows the fluorographic patterns of total and immunofinity-selected in vitro translation products, compared with the pattern of in vivo 35S labelled cryptogein. The antibodies raised against cryptogein detected in vitro-synthesized polypeptides of apparent Mᵣ 12,200. These immunoaffinity-isolated products had a mean Mᵣ in excess of 1850 compared with in vivo-labeled mature cryptogein. No immunoselected protein with a Mᵣ larger than 12,200 was observed.

Radiosequencing of the Cryptogein Signal Peptide

To determine whether the extra amino acids of the precursor of cryptogein were at the N-terminal position, immunoselected in vitro translation products were sequenced after
Figure 2. Electrophoretic patterns of cryptogein accumulated in the mycelium of *P. cryptogea* during culture. A, Coomassie blue-stained electrophoretogram of mycelium total protein extracts during growth; B, immunoblots of total protein extracts during growth. Lanes were loaded with approximately equal amounts of protein. The arrow indicates the migration position of mature cryptogein. Cry, cryptogein extracted from washed mycelium.

Radioactive labeling. When immunoselected *in vitro*-synthesized proteins were labeled with [35S]methionine, the maximal radioactivity was observed at the first degradation cycle and nowhere else. When these proteins were labeled with [35S] cysteine, the maximal radioactivity was observed at the 23rd degradation cycle (Fig. 4). After more than 20 Edman degradation cycles, the poor solubility of some degradation products of ATZ-cysteine gave rise to a rather broad peak of radioactivity. These results showed that 22 amino acids separate methionine and the first cysteine. No other methionine was found in the N-terminal sequence. The N-terminal end of these *in vitro*-synthesized proteins was found different from the mature cryptogein, in which no methionine was found before position 35 and the first cysteine at position 3.

**Characterization of mRNAs Directing Cryptogein Synthesis during Phytophthora Culture**

Total RNAs extracted from day 2 to day 28 mycelia were translated in a wheat germ system with [35S]methionine as the labeled amino acid. The fluorographic patterns of *in vitro* translation products (total and immunoaffinity isolated) are shown in Figure 5. Bands corresponding to immunoselected *in vitro*-synthesized products were clearly revealed from day 2 to 18 but were absent at later stages. Immunoselected products exhibited a *M*ₙ of about 12,200.

**DISCUSSION**

Results showed that the immune serum obtained after injection of purified cryptogein was monospecific; not only did it react with purified cryptogein but it detected only a
single band in total protein extracts of mycelium (Fig. 2). Immunoselected in vitro-synthesized products corresponded to a single band (Figs. 3 and 5B).

The difference between kinetics of appearance of cryptogein based on protein staining (Fig. 2A) and on immunodetection from western blots (Fig. 2B) came from the higher detection sensitivity of immunodetection. After 9 d of culture, the amount of secreted cryptogein and the dry weight of mycelium no longer increased. Secretion of cryptogein and growth of the mycelium stopped when Asn, the only nitrogen source in the culture medium, became limiting. Nevertheless, mRNAs isolated from mycelium were able to direct in vitro the synthesis of cryptogein until day 18. It appears that the massive secretion of elicitin stopped, although cryptogein was still accumulating in the mycelium (until day 14). Secretion began between day 2 and 4, later than biosynthesis did, and stopped earlier. From the comparison of electrophoretic patterns of total protein extracts of the mycelium with secreted cryptogein and N-terminal sequencing, it is concluded that cryptogein accumulated in the mycelium in its mature form.

Fluorograms showed that immunoaffinity-isolated translation products had a mean Mr in excess of 1850 compared with mature cryptogein. This size difference corresponds to a chain size difference of nearly 20 amino acids. This result suggests that the first cysteine (located at position 23) of the immunoselected in vitro-synthesized protein corresponds to the first cysteine (located at position 3) of the mature cryptogein. Thus, comparing fluorograms and radiosequence, cryptogein appeared to be synthesized with a 1850 D extra N-
terminal extension of 20 amino acids. This peptide contained a single methionine at position 1 and no cysteine. This extra N-terminal peptide is comparable in length with signal peptides described in the literature (21). No larger immunoselected protein was observed in in vitro translation products. Therefore, there is no long-term postranslational modification of the cryptogein polypeptide chain, such as cleavage of a preprotein. So, cryptogein was synthesized as a transitory preprotein with an extra peptide of 20 amino acids located in the N-terminal position and cotranslationally transformed into mature cryptogein, which accumulated in the mycelium in the processed form.

In conclusion, we demonstrated that cryptogein is synthesized as a preprotein undergoing the removal of a N-terminal peptide of 20 amino acids. This signal peptide removal occurs in the mycelium, i.e., before secretion of elicin into the culture medium. This leads us to conclude that cryptogein production by the fungus is performed in at least two steps in two compartments. Cryptogein is cotranslationally secreted initially into a cellular compartment isolated by a membrane, then exported subsequently to the exterior medium. Further studies will explore the mechanisms of this secretion at the cellular level.

No side chain alteration of cryptogein was observed except disulfide bond formation (15). The only postranslational modification is cotranslational and only concerns a signal peptide cleavage. The conformation of the mature protein should correspond directly to the primary structure (minus signal peptide) with a spontaneous folding. If the biologically active molecule is formed in this manner, it would be possible to expect a procaryotic vector to produce cryptogein, making feasible the engineering of elicin-like molecules, devoid of their necrotic properties, for protection against Phytophthora.

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LITERATURE CITED


