Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 849 (2007) 357–362
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification and characterization of cassiicolin, the toxin produced by *Corynespora cassiicola*, causal agent of the leaf fall disease of rubber tree☆,☆☆

Frédéric de Lamotte[a], Marie-Pierre Duviau[a], Christine Sanier[b], Robert Thai[c], Joël Poncet[d], Daniel Bieysse[b], Frédéric Breton[b], Valérie Pujade-Renaud[b,*]

[a] *INRA-UMR PIA - 2 Place Viala - Bat 31, 34 060 Montpellier cedex 01, France*
[b] *CIRAD, TA 80/03, Avenue Agropolis, 34398 Montpellier cedex 5, France*
[c] *C.E.A.- D.S.V.-D.I.E.P, Laboratoire de Biochimie des Protéines, Bât 152, Point Courrier 24, 91 191 Gif-sur-Yvette, France*
[d] *Institut de Génomique Fonctionnelle, CNRS UMR 5203, INSERM U661, Université de Montpellier 1, Université de Montpellier 2, 141 rue de la Cardonille, F-34094 Montpellier cedex 5, France*

Received 30 May 2006; accepted 27 October 2006
Available online 20 November 2006

Abstract

Cassiicolin, a phytotoxin produced by the necrotrophic fungus *Corynespora cassiicola*, was purified to homogeneity from a rubber tree isolate. The optimized protocol involves reverse phase chromatography followed by size exclusion chromatography, with monitoring of the toxicity on detached rubber tree leaves. Cassiicolin appeared to be a peptide composed of 27 amino acids, glycosylated on the second residue, with a N-terminal pyroglutamic acid and 6 cysteines involved in disulfide bonds. Its molecular mass was estimated to be 2885 Da. No significant sequence homology with other proteins could be found. The availability of pure toxin in sufficient amount is a prerequisite for its structure determination, which is a key step in the understanding of the aggression mechanism.

Keywords: Cassiicolin; *Corynespora cassiicola*; *Hevea brasiliensis*; Toxin; Purification; Chromatography; Amino acid sequence; Mass spectrometry

1. Introduction

Corynespora cassiicola (Berk. & Curt) Wei is a phytopathogenic fungus generating target necrotic spots not only on leaves but also on stems, roots, flowers or fruits of more than 70 plants species from tropical and subtropical countries [1,2]. *Corynespora* leaf fall disease (CLFD) is among the main pathologies affecting rubber tree (*Hevea brasiliensis*, Müll. Arg.). Both mature and immature leaves are affected, resulting in growth delay and decrease in rubber production. Severe infection leads to massive defoliation, and ultimately death of the affected trees. First reported in India and Malaysia [3,4], CLF disease has spread rapidly then and has become a limiting factor for the yield of natural rubber production in most Asian producing areas [5,6]. It is a constant threat for millions of rubber farmers in emerging nations as well as for developed countries which rely on natural rubber for health or transportation.

It soon appeared that a toxic substance was produced by the fungus, as crude filtrates from *C. cassiicola* cultures were able to induce disease symptoms on the host plant leaves [7,8]. Molecular characterization of this toxin is a key step toward the understanding of its action mechanisms and the development of defense strategies. Therefore, several attempts were made to purify the *C. cassiicola* toxin, from various isolates. A first attempt conducted on a *C. cassiicola* strain from *Mentha arvensis* [9] involved a series of Thin Layer Chromatography in various solvents and resulted in a partially purified product that could reproduce the symptoms of the disease. More recently, partial purification of the toxin was achieved from a *C. cassiicola* isolate from tomato, with a method involving one filtration

☆ This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann.
☆☆ The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number P84902 for Cassiicolin from *Corynespora cassiicola*.
* Corresponding author. Tel.: +33 4 67 61 71 27; fax: +33 4 67 61 57 93.
E-mail address: valerie.pujade-renaud@cirad.fr (V. Pujade-Renaud).

1570-0232/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2006.10.051

and three chromatographic steps [10]. Our previous investigations suggested that the toxin is a small protein and confirmed its causal role in CLF disease of rubber tree [11,12]. The toxin was named cassiicolin.

In this paper, we describe an optimized protocol allowing the purification of cassiicolin to complete homogeneity, which opens the door to its complete molecular characterization. The sequence and physical characteristics of the toxin extracted from a virulent *Corynespora cassiicola* isolate from rubber tree are presented.

2. Experimentals

2.1. Biological material

The susceptible *Hevea brasiliensis* clone PB260, from the CIRAD collection in Montpellier (France), was used for monitoring the toxicity of the purification fractions.

The *C. cassiicola* pathogenic strain CCP was collected on infected hevea leaves from Philippines and purified by single-conidium isolation. The isolate was maintained in the dark on Potato Dextrose Agar (PDA) medium at 25 °C. For long term conservation, mycelial plugs were kept in sterile water, in the dark, at room temperature. For toxin production, a liquid culture was set up in 500 ml flasks, by inoculating 100 ml of modified Czapeck medium (Saccharose 30 g l^{-1}, 2.2 g l^{-1} L-glutamic acid, K_2HPO_4 1 g l^{-1}, KCl 0.5 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}, 36 μM $FeSO_4 \cdot 7H_2O$, 35 μM $ZnSO_4 \cdot 7H_2O$, 40 μM $CuSO_4 \cdot 5H_2O$, pH being around 4 before sterilization) with 3 mycelial plugs (5 mm diameter) from a 7-days-old culture on PDA medium. The liquid culture was incubated without agitation at 25 °C (photoperiod 12 h) for 12–20 days. The medium was filtered once through Whatman filter paper to remove most of the mycelium, then twice through 0.22 μm Millipore membranes. The last filtration was conducted under sterile laminar flow. The sterile filtrate was stored at room temperature.

2.2. Bioassays

Hevea brasiliensis leaves (clone PB260, sensitive to *Corynespora cassiicola* strain CCP) in the morphogenetic stage D [13] were detached and placed on water-soaked filter paper in large Petri plates, abaxial side up. The lower epidermis was gently scraped (over 1 mm^2) using a scalpel blade and one drop of the tested sample (10 or 15 μl) was placed on the superficially wounded spot. Drops of chromatography buffers were used as negative controls, in appropriate dilution to, reproduce the solution of the tested sample after elution. The plates were maintained for 48–96 h at 25 °C (photoperiod 12 h) until symptoms were clearly visible.

2.3. Toxin purification

Chromatographic equipment: GE Healthcare (previously Amersham Biosciences) Akta Purifier 10 and Akta FPLC controlled by Unicorn v 5.10 were used for the chromatographic analysis.

2.3.1. Purification in acidic conditions

Capture: Capture of the toxin from the filtered culture was achieved by reverse phase chromatography (RPC) using GE Healthcare Source 15 RPC (1 cm × 8.5 cm). The column was equilibrated in Buffer A: H_2O, TFA 0.065%, acetonitrile 2%. Two hundred milliliters of culture filtrate were loaded onto the column. Elution was performed by a gradient from buffer A to buffer B (TFA 0.065%, acetonitrile 100%) as follows: 0–10% in 0.5 cv (column volume); 10–30% in 20 cv then 30–80% in 0.5 cv at 4 ml min^{-1}. Elution of the toxin was monitored by bioassay, after the neutralization of the samples (performed as described below).

Neutralization before bioassays: As the buffers used for purification were not compatible with the bioassay, samples (1 ml) of each fraction were concentrated (50%) in a Gyrovap (Howe, Banbury, UK) in order to evaporate the acetonitrile and then were neutralized with Tris–HCl 30 mM pH 8 before being tested on detached leaves as described above.

Concentration: Due to the low molecular weight of the toxin, a chromatography-based concentration method was chosen. This was performed on GE Healthcare Source 15 RPC (1 cm × 8.5 cm). The positive fractions were pooled, supplemented with 33% buffer A, loaded onto the column and eluted with a linear gradient (3 column volumes) from buffer A to buffer B. This step led to a threefold concentration. The highest yield of concentration obtained over various experiments was a tenfold concentration.

Size exclusion chromatography: After concentration, the positive fractions were loaded on a GE Healthcare Superdex 30 Prep-Grade (1.6 cm × 60 cm) equilibrated with $NaHPO_4$ 40 mM pH 7, acetonitrile 10% and eluted at 1 ml min^{-1}. Elution of the toxin was monitored by bioassay, without previous evaporation owing to the low acetonitrile concentration. The toxic fractions were concentrated again as described above.

2.3.2. Purification in physiological conditions

The same chromatographic techniques were used with pH 7 buffers. The culture filtrate was neutralized by adding K_2HPO_4 0.2 M. The two buffers used for RPC were Buffer C: KHPO4 10 mM pH 7 and Buffer D: id + 70% acetonitrile. No neutralization was required before bioassays but the samples were still slightly evaporated (to half of the initial volume) to eliminate the acetonitrile. The gradient used for capture was a linear gradient (20 column volumes) from buffer C to D.

2.4. Electrophoresis analysis

Samples were centrifuged for 10 min at 12,000 × *g* before being analyzed on 10–20% gradient Novex Tricine gels (Invitrogen Carlsbad, USA) and detected with silver Xpress staining kit (Invitrogen), according to the manufacturers recommendations. Molecular weight markers were SeeBlue Plus2 Prestained Standards from Invitrogen.

2.5. N-terminal sequencing

Removal of the pyroglutamate (pGlu) N-terminal blocking group in reduced condition: Four nanomoles of purified cassi-

icolin were pre-incubated at 70 °C for 60 min in a final volume of 20 μl of potassium phosphate buffer 50 mM pH 7.5, containing 1 mM EDTA and 10 mM DTT. Five microliters (1 mU) of PyroGlu-aminopeptidase (Sigma Cat No P-6236) were then added and the digestion proceeded for 2 h at 70 °C.

4-Vinyl-pyridine alkylation of Cys groups: After the cooling of the previous mixture to room temperature, 10 μl of 4-vinyl-pyridine (Sigma Cat No V-3877) at 86 mM were added. After 60 min incubation at room temperature, formic acid (0.5%) was added to terminate the reaction and the sample was desalted with C18 Zip-Tip Millipore (Bedford, MA, USA) system prior to sequencing.

N-terminal sequencing and identification of the alkylated Cys: Peptide sequencing was performed according to Edman chemistry on an automatic ABI 492HT/Procise peptide sequencer from Applied Biosystems (Foster City, CA, USA). Samples were loaded onto a TFA-treated glass fiber precycled with Biobrene Plus.

2.6. Mass spectrometry

Nanoelectrospray mass spectrometry was performed offline on a Quadrupole Time-Of-Flight (Q-TOF) mass spectrometer (QSTAR Pulsar-*i*, Applied Biosystems, Foster City, CA) fitted with a Protana nanospray inlet system (Protana, Odense, Denmark). Spectra were recorded using the Analyst QS software (Applied Biosystems). Parameters were adjusted as follows: ion spray voltage (IS), 900 V; curtain gas (CUR), 25; declustering potential (DP), 10–45 V; focusing potential (FP), 265 V; declustering potential 2 (DP2), 15 V. Fragmentation experiments (CID) were performed in the collision cell using nitrogen gas on the doubly or triply charged ions detected, with a collision energy profile optimized individually (20–65 V). Before being placed in the source tip holder, capillaries (Protana, Odense, Denmark) were loaded with the desalted samples according to a described procedure [14]. Briefly, a 2 μl aliquot of chromatrographically-purified cassiicolin was loaded on Poros 20 R2 (Applied Biosystems) packed in a gel-loader pipette tip and, washed with 1% formic acid (2 μl) and eluted with 2 μl of 50:50:1 methanol/water/formic acid.

3. Results

Cassiicolin was extracted from the filter-sterilized supernatant of a *C. cassiicola* culture in liquid medium. Heating the filtrate at 45 °C before loading onto the reverse phase chromatography column proved useful to reduce the viscosity and thus the back pressure. Two kinds of extraction conditions were tested, one using acidic chromatography buffers, which allowed direct processing of the naturally acidic (pH 4.5) crude filtrate, the other using neutral buffers, which required previous neutralization of the filtrate.

3.1. Purification in acidic conditions

As much as 200 ml of culture filtrate could be treated in a single capture run, at 4 ml min^{-1}. The profiles obtained were

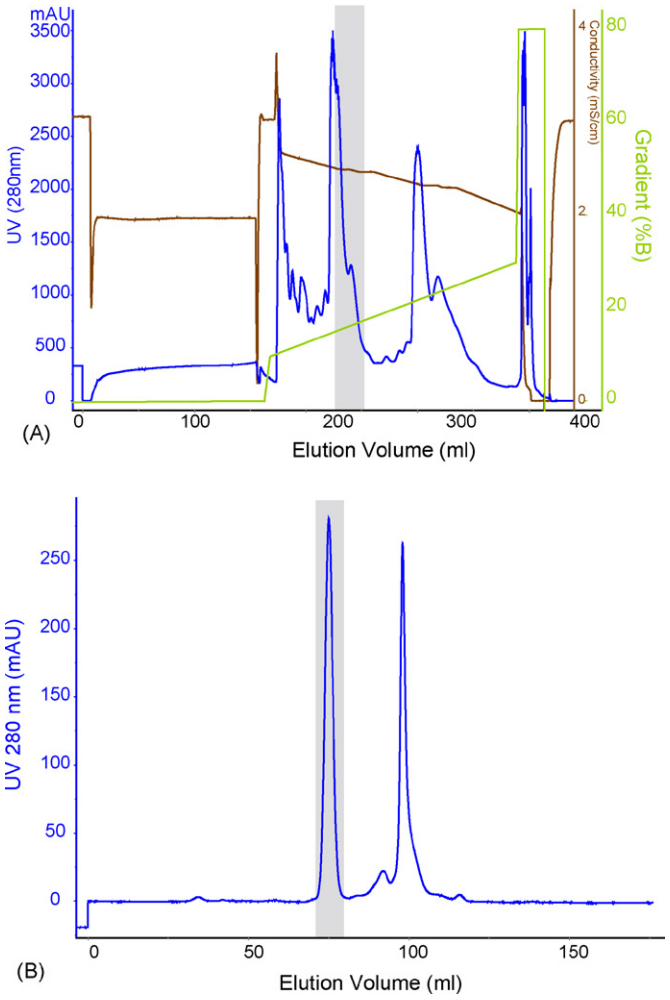

Fig. 1. Chromatograms corresponding to the purification of cassiicolin in acidic conditions. The toxic fractions identified by bioassays are indicated by shadowed areas. (A) Capture step by reverse phase chromatography using GE Healthcare Source 15 RPC. (B) Size exclusion chromatography on GE Healthcare Superdex 30 Prep-Grade columns.

reproducible (Fig. 1A). The toxicity of each fraction was tested on detached rubber tree leaves. Fractions exhibiting significant toxic activity (Fig. 2) were pooled, concentrated, and submitted to size exclusion chromatography, which resulted in two major peaks (Fig. 1B). The first major peak only, corresponding to an elution volume (EV) of 75 ml, appeared to contain the toxin, as evidenced by the bioassay (Fig. 2). The whole purification procedure was repeated 5 times to complete the purification of 1 l of culture filtrate, then the final toxin-containing fractions were pooled and lyophilized.

The toxin was analyzed on Tris–Tricine gels (Fig. 3). A single band corresponding to an apparent molecular mass of 15 kDa was observed in the ultimate positive fraction.

All attempts to quantify cassiicolin using classical spectrophotometric or colorimetric techniques remained unsuccessful, owing probably to the small size of the peptide and very low amount available. Knowing its amino acid composition, it was nevertheless possible to calculate its theoretical molar epsilon at 280 nm [15] and thus deduce the peptide's concentration in the final fraction from the measured absorbance at 280 nm, using

Fig. 2. Illustration of the bioassays. The samples tested and their ten-time dilutions were placed respectively on the left and right side of detached rubber tree leaves, after local scraping of the epidermis. Left leaf: positive reaction (toxic sample). Right leaf: negative reaction (non toxic sample).

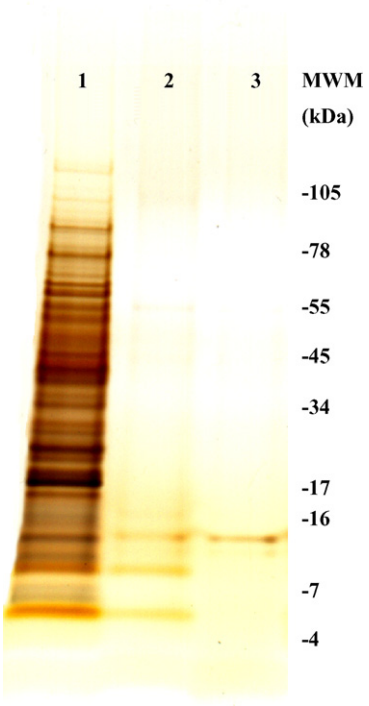

Fig. 3. Gel-electrophoresis and silver-staining of the samples. Lane 1: crude culture filtrate; lane 2, toxic fraction after capture by reverse phase chromatrography (GE Healthcare Source 15 RPC); lane 3: final purified product.

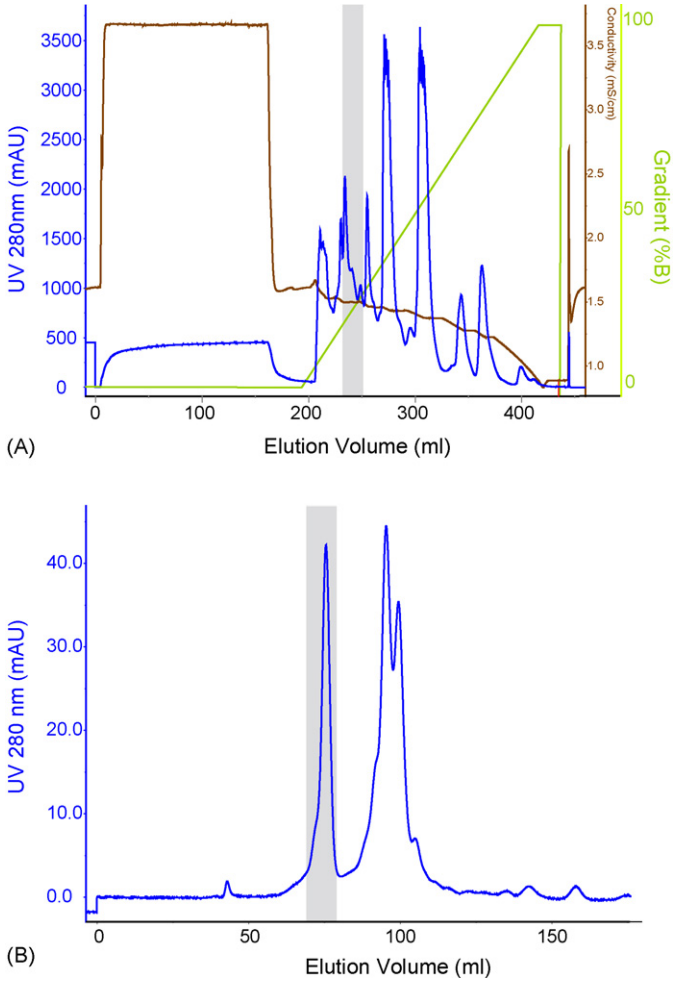

Fig. 4. chromatograms corresponding to the purification of cassiicolin in neutral conditions. The toxic fractions identified by bioassays are indicated by shadowed areas. (A) Capture step by reverse phase chromatography using GE Healthcare Source 15 RPC. (B) Size exclusion chromatography on GE Healthcare Superdex 30 Prep-Grade columns.

the Beer-Lambert law. The extraction yield estimated using this method was around 3 μmol of cassiicolin per litre of *C. cassiicola* culture filtrate.

3.2. Purification in neutral conditions

The neutralized culture filtrate was treated similarly, except that the chromatography buffers were at pH 7. The chromatograms obtained after capture by reverse phase chromatography (Fig. 4A) were significantly different compared to the profiles obtained with the protocol in acidic conditions (Fig. 1A). The fractions identified as toxic by the bioassays were pooled, concentrated and submitted to size exclusion chromatography, which generated three major peaks (Fig. 4B). Only the first major peak, corresponding to an elution volume (EV) of 75 ml, was found to contain the toxin, as evidenced by the bioassay (Fig. 2). The toxic fractions were pooled and lyophilized as described previously.

Analysis of the purified toxin on Tris–Tricine gels was identical to the previous one (Fig. 4).

The estimated yield of extraction, calculated over several experiments, was in the range of 1–6 μmol of cassiicolin per litre of *C. cassiicola* culture filtrate.

3.3. Molecular mass determination

The purified toxin was analysed by ESI-QTOF for accurate molecular mass determination. When declustering potential (cone voltage) was set to 45 V, two products with monoisotopic mass of 2884.96 and 2708.87 Da could be observed as doubly charged (*m*/*z* 1443.46 and 1355.43 Da) or triply charged (*m*/*z* 962.66 and 903.98 Da) ions. By diminishing DP to its lower possible value (10 V), the lower mass peak disappeared almost completely, leaving the higher mass peak only. This indicated that the light form was probably generated by an in-source collision-induced dissociation process from the main heavy form (176 Da neutral loss).

Attempts to fragment ions at either *m*/*z* 1443.46 or *m*/*z* 962.66 by collision-induced dissociation (CID MS–MS) led only to the 176 Da neutral loss, followed by successive 18 Da or 17 Da neutral losses. This 176 Da moiety detached by CID MS–MS is in the molecular mass range of sugars such as hexuronic acids. The 2708.87 Da peptide may therefore be a deglycosylated form of the toxin. Mass spectrometry analysis gave identical results whatever the extraction protocol used (acidic versus neutral conditions).

3.4. Amino acid sequence determination

As direct peptide N-terminal sequencing by the Edman technique initially failed, digestion with a pyroglutamate-aminopeptidase was attempted to remove a potential N-terminal blocking pyroglutamate group. Edman sequencing could then be achieved, after reduction and alkylation of the cysteine groups. Cassiicolin thus appeared to be a 27 amino acids peptide, with 6 cysteinyl residues as unambiguously detected after TCEP reduction and 4-VP alkylation of the cystein groups. The PTH-derivative of the second amino acid eluted in two specific peaks, unidentified when compared to the 20 classical standard amino acids. This indicates the presence of a modified amino acid in position 2, potentially bearing a post-translationally added moiety that may be partially degraded by the sequencing chemistry, generating two unidentified peaks. It is unlikely that two forms of the toxin may coexist in the initial sample as both RP-HPLC (data not shown) and mass analysis in mild conditions generated a single peak, indicating that the cassiicolin sample was homogeneous. NMR-analysis of the purified cassiicolin structure allowed identifying this second residue as a threonine bearing a mannose moiety methylated on its C3 position [in preparation].

The full Cassiicolin amino acid sequence is as follows: PyroGlu- T* - C V S C V N F G N G F C G D N C G N S W A C S G C, with T* being the glycosylated threonine (accession number P84902). No significant sequence homology could be found using the NCBI BLAST program.

3.5. Other physical properties

A 1 μg ml^{-1} solution of purified cassiicolin retained its full toxicity after being autoclaved for 30 min at 120 °C, indicating that it was extremely heat-stable. The cassiicolin toxicity was also independent of pH: no significant difference in toxicity was observed after incubating a 1 μg ml^{-1} solution of purified cassiicolin (for 12 h at 4 °C) at pH 4, 6 or 8.

Reduction and alkylation of the toxin lead to a total loss of toxicity.

The isoelectric point calculated from the aminoacid sequence varied from 2.4 to 2.8 depending on the method of calculation applied.

4. Discussion

We have set up an optimized protocol for the purification of cassiicolin, the toxin responsible for *C. cassiicola* Leaf Fall Disease symptoms on rubber tree. Cassiicolin was extracted from the filter-sterilized supernatant of a *C. cassiicola* culture (strain CCP). This is the first report of the purification of cassiicolin to homogeneity. Although the toxin was highly diluted in a complex culture medium, we could capture it without any preparation step taking advantage of the high capacity of the reverse phase chromatography together with its tolerance to salt. The high selectivity of the source 15 RPC beads (15 μm porosity) also greatly contributed to the good purification yield obtained upon this first chromatographic step. Purification was then completed by size exclusion chromatography. Bioassays on detached leaves proved to be a precious tool for monitoring the toxicity of the fractions.

The successful purification of cassiicolin allowed us to achieve its biochemical characterization. As the filtrate was naturally acidic (pH 4), we first set up a protocol to maintain such acidic environment. In a second phase, a modified protocol in neutral conditions was set up, with the idea to mimic the plant cell environment and to preserve putative features of the toxin such as sugar chains that may be altered by the highly acidic purification conditions. Mass spectrometric analysis demonstrated that both extraction procedures resulted in the same final product, although the intermediate chromatographic profiles were different. Cassiicolin was therefore not affected by the acidic environment in the first protocol and it can be assumed that the purified toxin conserved all its physiological attributes.

The estimated yield of extraction was in the same range for both protocols. A lower number of runs was required with the first protocol in acidic conditions because no dilution of the crude filtrate was necessary. However, the second protocol in neutral conditions presents other technical advantages such as the possibility to avoid neutralization of the fractions before the bioassays.

The molecular mass of cassiicolin was found to be 2884.96 Da, as determined by mass spectrometry. In-source fragmentation experiments demonstrated that a neutral 176 Da moiety, potentially a sugar (hexuronic acid), could be easily detached. Fragmentation of glycosidic groups at the ESI source is frequent [16]. This is coherent also with the Edman microse-

quencing data detected two unidentified peaks in position 2, suggesting a modified amino acid and its partially degraded form as a consequence of the Edman chemical reactions.

Cassiicolin appears to be a peptide of 27 residues that exhibits two post-translationally modified residues: a N-terminal pyroglutamatic acid and a threonine with a single methyl-mannose in the second position (manuscript in preparation). All 6 cysteines of the sequence are likely involved in disulfide bonds as the calculated monoisotopic mass agrees with the experimental mass of the peptide supposing the presence of 3 disulfide bonds and one sugar moiety. The discrepancy between the molecular mass measured by spectrometry and the apparent mass observed by gel electrophoresis may suggest a quaternary structure formed by aggregation of several 27 amino acids subunit. However, it may also be explained by migration artifacts due to the very small size and specific structure of the peptide as well as its very acidic isoelectric point. The 3D structure created by the three disulfide bonds seems crucial for the toxicity of cassiicolin as reduction and alkylation resulted in a complete loss of toxicity.

Many fungal phytotoxins have been described previously (for a review see: [17–19]). A majority of them are non-selective, as they affect a broader range of plant species than the producing fungus infects. On the other hand, host-selective toxins (HSTs) appear to be specific to an individual plant cultivar and are primary determinants of the host range. Most of the phytotoxins described so far are secondary metabolites of diverse structure including polyketides, cyclic peptides, terpenoids, saccharides or other compounds of unknown biogenesis. The Ptr-toxins A and B from *Pyrenophora tritici-repentis* are the only examples of ribosomally-synthesized host-selective toxins characterized so far [20–24].

Cassiicolin appears to be a new peptide toxin sharing neither sequence homology with the previously described proteinous HSTs nor with any other peptide or protein.

A complete description of cassiicolin 3D-structure may bring clues concerning its action mechanisms, by comparison with the 3D-structure of previously described active molecules. Following our extraction procedure, a sufficient amount of pure cassiicolin could be obtained to fit the requirements of an NMR analysis, with the objective to determine the full 3D-structure and post-translational modifications of cassiicolin [in preparation]. Investigating the role of the post-translational modifications in the toxicity of the toxin will also be an interesting perspective. Post-translational modifications are known to play a key role in the activity of some toxic molecule. As an example, deglycosylation of a phytotoxin associated with sheath blight disease in rice, *via* an extracellular α-glucosidase from *Trichoderma viride*, resulted in the inactivation of the toxin [25].

The availability of purified cassiicolin opens other interesting perspectives. For example, purified cassiicolin may prove to be a useful tool for the identification of its own target in the plant cells. In addition, triggering the disease with purified toxin rather than spore inoculation offers the advantage to avoid contamination of the plant material with fungal material in functional genomic programs aiming at the identification of plant genetic resistance factors. Mass production of pure cassiicolin will also find applications in rubber tree selection, for the easy screening of progenies for their resistance to *C. cassiicola* [12].

Acknowledgements

We are grateful to Dr Philippe Barthe whose contribution in NMR analysis of the cassiicolin (manuscript in preparation) permitted to work out ambiguities concerning the toxin amino acid sequence. We would also like to thank Véronique Roussel for maintaining the *C. cassiicola* collection and Gérald Oliver for taking care of the rubber plants in the green house. The authors would like to dedicate this work to Pr. d'Auzac for his faithful support and encouragements.

References

[1] W.P.K. Silva, D.S. Multani, B.J. Deverall, B.R. Lyon, Aust. J. Bot. 43 (1995) 609.
[2] W.P.K. Silva, R.L.C. Wijesundera, E.H. Karunanayake, C.K. Jayasinghe, U.M.S. Priyanka, Plant Dis. 84 (2000) 202.
[3] A. Newsam, Plant Pathology Division Report, Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia, 1960, p 63.
[4] T. Ramakrishnan, P. Pillay, Rubber Board Bull. 5 (1961) 52.
[5] M.J. Manju, S.P. Idicula, C.K. Jacob, K.K. Vinod, E.E. Prem, M. Suryakumar, R. Kothandaraman, Indian J. Nat. Rubber Res. 14 (2001) 137.
[6] W.P.K. Silva, B.J. Deverall, B.R. Lyon, Plant Pathol. 47 (1998) 267.
[7] N.I.S. Liyanage, A.S. Liyanage, J. Rubber Res. Inst. Sri Lanka 65 (1986) 51.
[8] P. Onesirosan, C.T. Mabuni, R.D. Durbin, R.B. Morin, D.H. Rich, D.C. Arny, Physiol. Plant Pathol. 5 (1975) 289.
[9] R.S. Shukla, K.P. Singh, J. Med. Aromat. Plant Sci. 19 (1997) 682.
[10] S. Kurt, Turk. J. Agric. Forest 28 (2004) 389.
[11] F. Breton, Ph.D. thesis, University of Montpellier 2, Montpellier, 1997.
[12] F. Breton, C. Sanier, J. d'Auzac, J. Rubber Res. 3 (2000) 115.
[13] F. Hallé, R. Martin, Adansonia 8 (1968) 475.
[14] M. Wilm, M. Mann, Anal. Chem. 68 (1996) 1.
[15] C. Combet, C. Blanchet, C. Geourjon, G. Deléage, Trends in Biochem. Sci. 25 (2000) 147.
[16] A. Gonzalez de Peredo, D. Klein, B. Macek, D. Hess, J. Peter-Katalinic, J. Hofsteenge, Mol. Cell Proteomics 1 (2002) 11.
[17] A.E. Desjardins, T.M. Hohn, Mol. Plant Microbe Interact. 10 (1997) 147.
[18] J.D. Walton, Plant Cell 8 (1996) 1723.
[19] T.J. Wolpert, L.D. Dunkle, L.M. Ciuffetti, Annu. Rev. Phytopathol. 40 (2002) 252.
[20] G.M. Ballance, L. Lamari, C.C. Bernier, Physiol. Mol. Plant Pathol. 35 (1989) 203.
[21] S.E. Strelkov, L. Lamari, G. Ballance, Mol. Plant Microbe Interact. 12 (1999) 728.
[22] A. Tomas, G. Feng, G. Reech, W. Bockus, J. Leach, Mol. Plant Microbe Interact. 3 (1990) 221.
[23] R.P. Tuori, T.J. Wolpert, L.M. Ciuffetti, Mol. Plant Microbe Interact. 8 (1995) 41.
[24] H.-F. Zhang, L. francl, J. Jordahl, S. Meinhardt, Phytopathology 87 (1997) 154.
[25] V. Shanmugam, S. Sriram, S. Babu, R. Nandakumar, T. Raguchander, P. Balasubramanian, R. Samiyappan, J. Appl. Microbiol. 90 (2001) 320.