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Cercospora beticola toxins III. Purification, thin-layer and high-performance liquid chromatographic analyses

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Abstract

Cercospora beticola toxins were extracted from the mycelium and purified by flash chromatography and crystallization. TLC was used to monitor the purification. The pure compounds were used to determine the optimum conditions for HPLC analysis. The HPLC method was used to quantify these metabolites in crude extracts.

1. Introduction

The phytopathogenic fungus *Cercospora beticola* is responsible for the main leaf spot disease on sugar beet named cercosporiose. It produces coloured secondary metabolites with complex structures: (i) a red compound, called cercosporin (Fig. 1), is a perylenequinone [1,2], a photodynamically activable compound that can induce lipid peroxidation and membrane damage [3], and (ii) bright yellow compounds, one of them previously known as CBT (*Cercospora beticola* toxin). Only a few data concerning the chemical structure of CBT have been published during the past 40 years [4,5], whereas the biological activities have been widely described [6–10]. We are interested in its inhibitory effect on plant membrane H^+-ATPases and the determination of the structure was essential to pursue this research. Therefore, we isolated the six major yellow compounds from a crude mycelial extract of a *C. beticola* strain. We named them beticolins and we proposed structures for five of the compounds [11–14]. Two of them (beticolin 2 and 4) have been determined by X-ray diffraction analysis (Fig. 1). They represent the leading members of xanthraquinones, a new class of natural toxins which can be divided into two sub-classes according to the type of cyclization of the heterocycle: the cyclization could occur with the oxygen being on the *ortho* position to the chlorine atom (beticolin 2 or 4, Fig. 1) or with the oxygen on the *para* position (beticolin 1 or 3, Fig. 1).

In this work, the purification and TLC analyses of the toxins are reported, together with an HPLC method for qualitative and quantitative analyses of these toxins. The results show that this HPLC method is suitable and convenient for

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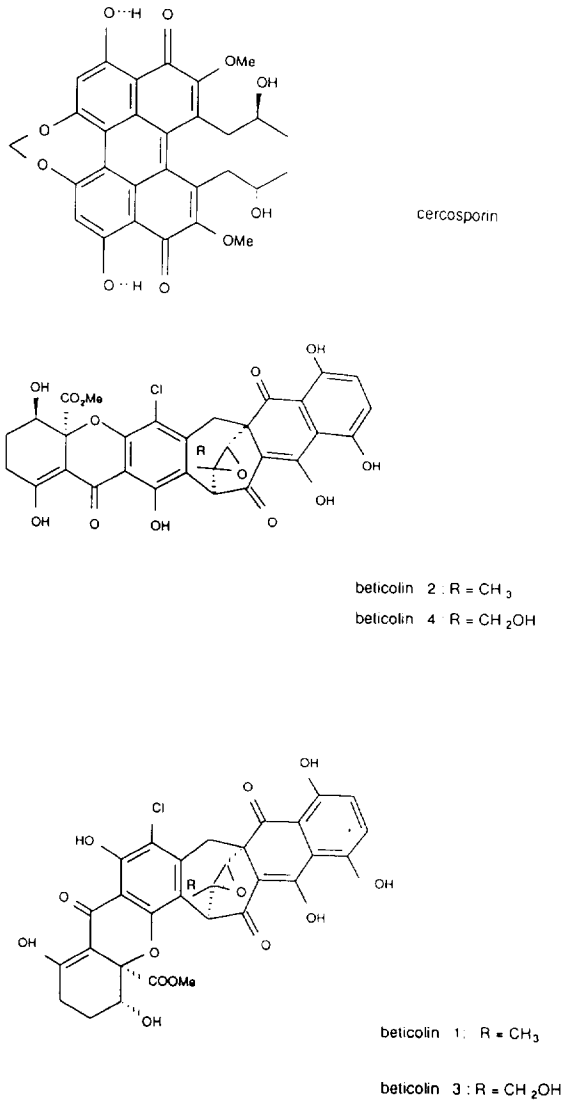

Fig. 1. Structures of some secondary metabolites produced by *C. beticola*.

analysing the production of *C. beticola* secondary metabolites.

2. Experimental

2.1. Organism and culture conditions

A *Cercospora beticola* isolate (CM) from the Laboratoire de Pathologie Végétale, INRA de Colmar (France), was used. It was cultured on V8 (Campbell's) medium diluted fivefold in Roux flasks at 25°C under constant light (21.5 μM/m^2; 1500 lux).

2.2. Extraction of toxins

The mycelium was removed from the culture medium by filtration and ground in a Waring blender in ethyl acetate (20 ml/g of fresh mycelium). After filtration, the extraction was repeated until the organic extract was colourless. The organic extracts were pooled, washed with water, dried with sodium sulfate and evaporated in a vacuum rotary evaporator.

2.3. Purification of toxins

The crude extract (5 g) was chromatographed (flash chromatography) on pretreated 60-μm silica gel (SDS, Villeurbanne, France). The pretreatment consisted of mixing silica gel (200 g) with an aqueous solution (500 ml) of phosphoric acid (10 g) and calcium bis(dihydrogenphosphate) (20 g), then the mixture was dried at 120°C as described by Balis and Payne [15]. The column was equilibrated with chloroform and the toxins were eluted using a chloroform–ethyl acetate gradient (150 ml for each fraction) from 10 to 100% ethyl acetate. The composition of each eluted fraction (30 ml) was monitored using three TLC systems: (I) (CMA) chloroform–methanol–acetic acid (100:2:1) and pretreated plates (Merck 60 F-254); the pretreatment consisted of dipping the plates in an aqueous solution of phosphoric acid and calcium bis(dihydrogenphosphate) with the composition described above and reactivating them by heating at 120°C; (II) (Hex/EA) hexane–ethyl acetate (1:1) and pretreated plates; (III) (*CMW) chloroform–methanol–water (100:20:2) and regular plates (Merck 60 F-254). All the eluted fractions which contained the same beticolin as the major compound were pooled, washed with water, dried with sodium sulfate and evaporated in a vacuum rotary evaporator. Fractions containing the same beticolin but showing contamination were pooled, concentrated and rechromatographed on a silica gel column as described

above. The toxins were crystallized from ethyl acetate–hexane, repeatedly if necessary, until their melting points became constant and homogeneity in TLC was obtained. These compounds were used as standards for HPLC analysis and for structure elucidation.

2.4. Reagents

The solvents used for extraction, purification (analytical-reagent grade) and HPLC (HPLC grade) and the silica gel used for flash chromatography were purchased from SDS. Water was deionized with a Milli-Q system (Waters–Millipore, St. Quentin en Yvelines, France). TLC plates were obtained from Merck (Nogent sur Marne, France).

2.5. Chromatographic columns and equipment and LC analyses

A 5-μm Ultrasphere ODS column (25 × 2 mm I.D.) from Beckman (Gagny, France) was used. HPLC analyses were performed with a Beckman system consisting of a Model 126 programmable solvent module, a Rheodyne syringe-loading sample injector equipped with a 5-μl sample loop and a Model 481 variable-wavelength detector set at 340 or 255 nm (Waters–Millipore), monitored by a Dell computer using System Gold software (Beckman).

The optimum gradient profile was as follows: initial conditions, acetonitrile–water containing 5% acetic acid (50:50); 0–8 min (70:30); 8–13 min (85:15); 13–16 min (100:0); 16–22 min (100:0); 22–27 min (50:50); linear steps; eluent flow-rate, 0.25 ml/min.

3. Results and discussion

3.1. Purification of toxins and TLC analyses

C. beticola secondary metabolites were extracted from the mycelium. The purification of these compounds, which was achieved by flash chromatography and crystallization, was monitored by TLC. For the flash chromatography, by pretreating the silica gel with phosphoric acid and calcium bis(dihydrogenphosphate), as described by Balis and Payne [15], the elution of the toxins could be obtained with less polar solvent mixtures than with non-treated silica gel and the eluted fractions could be washed with water. These conditions avoid degradation during the chromatographic steps (elution and concentration of the eluted fractions). It allowed us to obtain cercosporin and six major yellow compounds in larger amounts (a few milligrams) and with good reproductibility. The pretreatment of the TLC plates induced effects comparable to those obtained for the silica gel in flash chromatography: Migration was facilitated by the pretreatment. It can be observed from Table 1 that 2% of methanol in the eluting mixture is needed for the pretreated plate whereas 20% is needed for untreated plates to obtain similar R_F values. Similar conclusions can be drawn by comparing, using the same eluting system, treated with non-treated TLC plates (CMW/*CMW). Moreover, three TLC systems were used to analyse the eluted fraction in order to detect possible co-migration (CMA, Hex/EA and *CMW). TLC analyses were useful for monitoring the homogeneity of the samples during the purification steps, as well as the

Table 1
R_f values of *C. beticola* metabolites under different TLC conditions

Compound	CMA	Hex/EA	*CMW	CMW
Beticolin 0	0.49	0.38	0.48	0.82
Beticolin 1	0.22	0.27	0.26	0.77
Beticolin 2	0.46	0.32	0.46	0.82
Beticolin 3	0.13	0.10	0.12	0.69
Beticolin 4	0.22	0.18	0.23	0.72
Beticolin 7	0.22	0.24	0.48	0.70
Cercosporin	0.03	0.07	0.82	0.82

C. beticola metabolites were analysed using different TLC systems:
TLC plates treated with phosphoric acid and calcium bis-(dihydrogenphosphate) and three elution mixtures: CMA = chloroform–methanol–acetic acid (100:2:1); HEX/EA = hexane–ethyl acetate (1:1); CMW = chloroform–methanol–water (80:20:2); *CMW = chloroform–methanol–water (80:20:2) with regular plates (untreated).

melting points (data not shown). To analyze these metabolites with better accuracy and to determine them in crude extracts, an HPLC method was required.

3.2. HPLC analyses of standards

Each toxin was analyzed by HPLC using UV detection under different conditions: diol phase with a gradient of dichloromethane–ethyl acetate, hexane–ethyl acetate; NH_2, C_8 or C_{18} phases with acetonitrile–water containing acetic acid. The latter conditions gave the best results and the gradient step profile was then optimized: the percentage of acetic acid, the initial acetonitrile percentage and the slopes of the different steps were modified in order to select the gradient with the shortest duration and the best resolution. The chromatogram obtained with a reconstituted mixture of the major *C. beticola* metabolites is shown in Fig. 2 with the elution profile of the gradient. In Table 2, the retention times are reported for the toxins measured individually and together in a reconstituted mixture. The detection limit for each compound (corresponding to ten times the noise level) with detection at 340 nm is given in Table 2. Analyses were also performed at 255 nm, which allows a more sensitive detection of cercosporin. The molar absorptivities (ε_M) for the two λ are

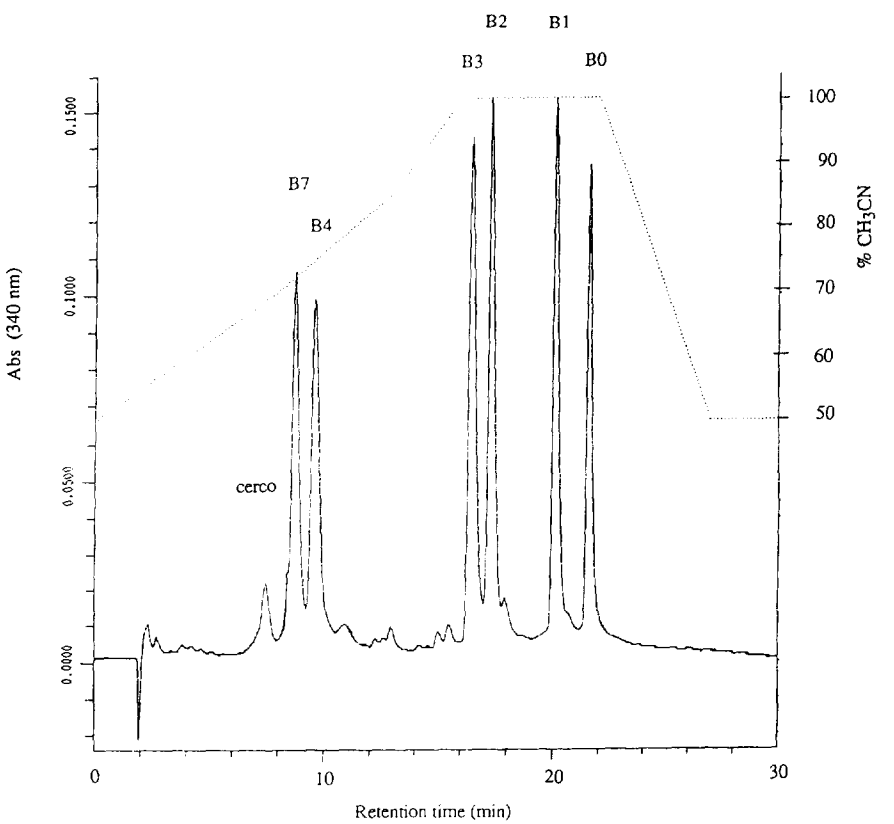

Fig. 2. Chromatogram of a reconstituted mixture of *C. beticola* major metabolites: 5 μl of a solution containing 0.25 μg of beticolin 0 (B0), 0.25 μg of beticolin 2 (B2), 0.28 μg of beticolin 4 (B4), 0.32 μg of beticolin 7 (B7), 0.25 μg of beticolin 1 (B1), 0.25 μg of beticolin 3 (B3) and 0.125 μg of cercosporin (cerco) under the following conditions: 5-μm Ultrasphere ODS HPLC column (25 × 2 mm I.D. and an eluent gradient: initial conditions, acetonitrile–water containing 5% acetic acid (50:50); 0–8 min (70:30); 8–13 min (85:15); 13–16 min (100:0); 16–22 min (100:0); 22–27 min (50:50); linear steps; eluent flow-rate, 0.25 ml/min; UV detector set at 340 nm.

Table 2
Retention times, molar absorptivities ($\varepsilon_{M\ 255\ nm}$, $\varepsilon_{M\ 340\ nm}$) and detection limits for *C. beticola* metabolites

Parameter	Cercosporin (M_r = 534)	Beticolin 7 (M_r = 656)	Beticolin 4 (M_r = 654)	Beticolin 3 (M_r = 654)	Beticolin 2 (M_r = 638)	Beticolin 1 (M_r = 638)	Beticolin 0 (M_r = 622)
$t_R \pm \sigma_{n-1}$ (min)	7.49 ± 0.10	8.81 ± 0.10	9.64 ± 0.08	16.46 ± 0.05	17.31 ± 0.06	20.04 ± 0.05	21.52 ± 0.10
$\varepsilon_{M\ 255\ nm}$	21500	17300	18200	14000	17400	29600	15300
$\varepsilon_{M\ 340\ nm}$	4800	19400	29100	29000	28200	28500	23400
Detection limit (ng)[a]	20	5	5	5	5	5	5

[a] The HPLC analyses were performed according to the conditions described for Fig. 2. Detection limits correspond to ten times the noise level; the detector was set at 340 nm.

reported in Table 2. Calibration graphs for these compounds were obtained by plotting the peak area against the amount injected. Good linearity was obtained between 0.01 and 1 μg (Table 3).

3.3. Quantitation of *C. beticola* metabolites in crude extracts

The same method was used to analyse crude samples in order to determine qualitatively and quantitatively the fungal secondary metabolite production. Fig. 3 shows a typical chromatogram of a crude mycelial extract obtained from an isolate of *C. beticola* (CM) grown in vitro for 11 days. By comparing the retention times of the peaks present on the two chromatograms (Figs. 2 and 3) we could identify the major compounds and quantification was achieved using the calibration graphs. The amount of each compound in the crude extract is shown in Table 4. It corroborates the evaluation we made for the composition of the crude extract after the large-scale purification (flash chromatography) and analysis of the TLC plates (data not shown). Work is in progress in order to obtain the minor compounds in sufficient amounts to determine their structures and analyse their biological activities.

Table 3
Linearity of the response in HPLC analyses

Compound	No. of analyses	r^2	a
Beticolin 0	8	0.999	185
Beticolin 1	9	0.998	189
Beticolin 2	8	0.999	204
Beticolin 3	8	0.997	167
Beticolin 4	8	0.993	197
Beticolin 7	8	0.997	187
Cercosporin	10	0.998	59

Linearity responses were determined from the HPLC analyses (under the conditions described for Fig. 2) by expressing the relationship between the area under each peak and the amount injected. Linearity was obtained between 0.01 and 1 μg.

4. Conclusion

Purification of *C. beticola* secondary metabolites under the conditions described here enabled us to obtain compounds with complex structures. TLC analyses were useful for monitoring the purity and stability during the different purification steps. The proposed HPLC method is convenient for the analysis of *C. beticola* metabolites. As a complement to TLC analysis, it represents a valuable tool in the study of secondary metabolite production of *C. beticola* isolates in vitro without prepurification of the crude extract. It should allow us to determine whether there is a correlation between the production of one of these products and pathogenicity of the producing isolate. We should also be able to isolate the minor compounds, which were difficult to obtain by flash chromatography, in reasonable amount and purity. Analyses of crude extracts using LC–MS will also be considered.

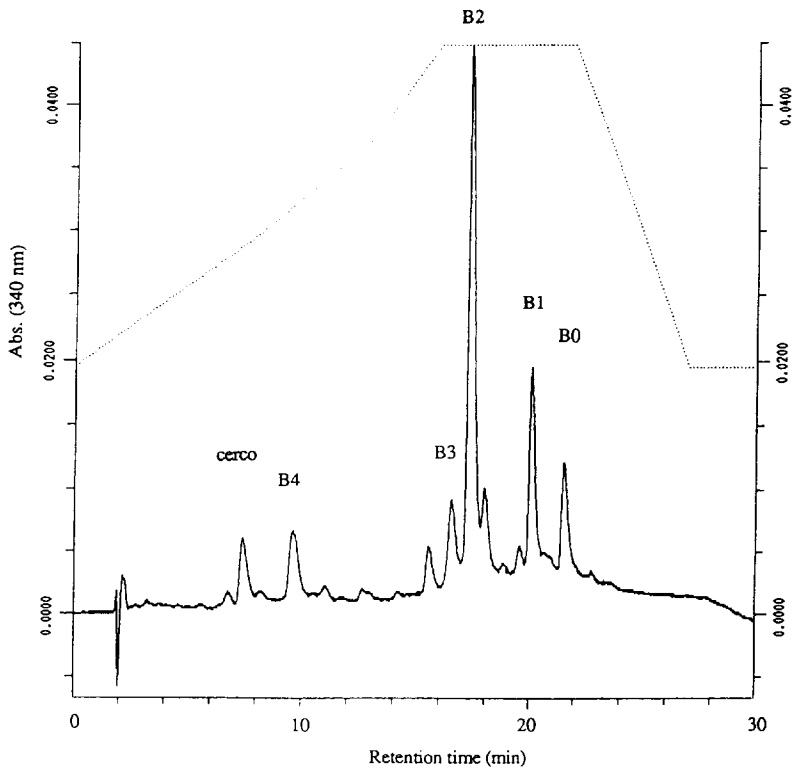

Fig. 3. Chromatogram of a crude mycelial extract obtained from an 11-day old culture of the *C. beticola* isolate CM grown on V8 medium (injection: 5 μl). HPLC conditions as in Fig. 2.

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Table 4
Contents of a *C. beticola* crude mycelial extract

Compound	Amount (μg)
Cercosporin	270 ± 5
Beticolin 4	140 ± 5
Beticolin 3	134 ± 3
Beticolin 2	766 ± 7
Beticolin 1	250 ± 5
Beticolin 0	180 ± 5

The *C. beticola* isolate (CM) was grown for 11 days on 50 ml of V8 medium. Each value (total amount for a 50-ml culture) represents the means of four replicates and was calculated from chromatograms, one of which is shown Fig. 3. The areas under the peaks corresponding to the identified beticolins were calibrated against standards.

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