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Studies on the dynamics of the production of destruxins by *Metarhizium anisopliae* Direct high-performance liquid chromatographic and fast atom bombardment mass spectrometric analysis correlated with biological activity tests

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Abstract

In this work, the new strain Ma23 of the fungus *Metarhizium anisopliae* is demonstrated to be an effective source of destruxins, known insecticidal cyclodepsipeptides, under optimized culture conditions. The dynamics of the destruxin production is analysed by fast atom bombardment MS and HPLC, using wide-pore reversed-phase packings without any pretreatment of the culture media samples. By this approach, we show that twelve different destruxins are produced by *M. anisopliae*. The most abundant toxins have been unequivocally identified and HPLC quantitation shows that E destruxin, the most toxic compound of the series, accounts for ~35–40% of the whole toxin production from Ma23. A good correlation between the pathogenic effect of the fungal samples and amounts of destruxins produced is also demonstrated with an optimum of toxicity after four days.

Keywords: Metarhizium anisopliae; Destruxins; Toxins

1. Introduction

The filamentous fungus *Metarhizium anisopliae* is pathogenic for several species of insects, including Lepidoptera [1]. This pathogen produces hexacyclodepsipeptidic toxins of the group of destruxins, the most biologically active being E destruxin (E-DTX) (Fig. 1). The DTXs, when applied by injection and per os, show an insecticidal activity and in lepidopterans such as *Galleria mellonella* larvae, they also induce a typical flaccid paralysis following a brief tetanisation, the intensity and duration of which is dose-dependent [2–5].

Up to now, several strains of *M. anisopliae* have been studied and the main objective of those studies was the isolation of new active natural toxins [3–7] which resulted in the structural characterization of more than twenty destruxins. In contrast, there is no published paper about the dynamics and the mechanisms of destruxin biosynthesis.

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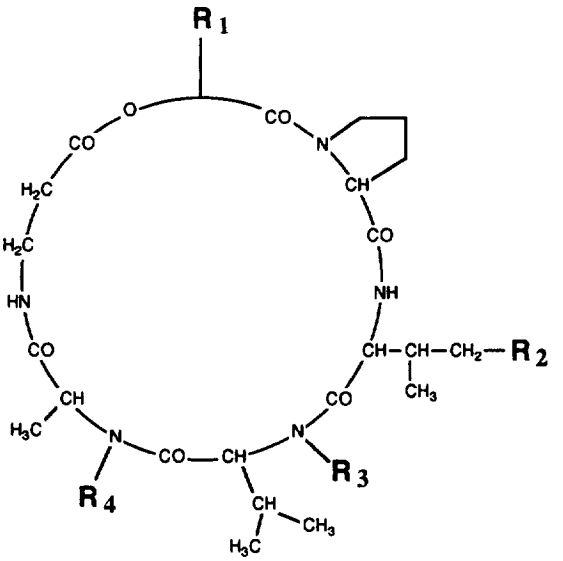

Destruxins	R_1	R_2	R_3	R_4	MW
Ed	—CH_2—CH(OH)—CH_2OH	-CH_3	-CH_3	-CH_3	611
D	—CH_2—$CH(CH_3)$—CO_2H	-CH_3	-CH_3	-CH_3	623
E	—CH_2—CH(O)—CH_2	-CH_3	-CH_3	-CH_3	593
C	—CH_2—$CH(CH_3)$—CH_2OH	-CH_3	-CH_3	-CH_3	609
A	—CH_2—CH=CH_2	-CH_3	-CH_3	-CH_3	577
B	—CH_2—$CH(CH_3)_2$	-CH_3	-CH_3	-CH_3	593
E_2	—CH_2—CH(O)—CH_2	H	-CH_3	-CH_3	579
C_2	—CH_2—$CH(CH_3)$—CH_2OH	H	-CH_3	-CH_3	595
A_2	—CH_2—CH=CH_2	H	-CH_3	-CH_3	563
B_2	—CH_2—$CH(CH_3)_2$	H	-CH_3	-CH_3	579
Chl	—CH_2—CH(OH)—CH_2Cl	-CH_3	-CH_3	-CH_3	630
desMeB	—CH_2—$CH(CH_3)_2$	-CH_3	H	-CH_3	579
ProtoB	—CH_2—$CH(CH_3)_2$	-H	H	H	565

Fig. 1. Structure of destruxins.

In this work, our purpose was to follow the dynamics of the destruxin production by the highly toxic strain Ma23 under culture conditions previously optimized. We studied which DTXs can be produced and tried to estimate the concentrations of the toxins at various stages of fungal culture development. To this end we examined samples of medium and mycelium of the strain Ma23.

The originality of this research consists of:

(1) Studying the dynamics of destruxin production by the strain Ma23,

(2) The use of complementary analytical techniques which give quantition aspects and structural informations. Thus, we employ HPLC with wide-pore packings and fast atom bombardment mass spectrometry (FAB–MS). Those techniques allow the direct analysis of exogenous compounds in biological samples without prior extraction as already described [8–11]. Thus, tedious pretreatment can be avoided. To conclude about the advantages of the 'direct analytical approach' for fungal study, it has been compared to the 'classical' one which involves extractions before analysis,

(3) Performing, in parallel with these analytical studies, bioassays based on the determination of the paralysis rate induced in *Galleria mellonella* by injection of culture media collected after different time intervals. We search to determine if a good correlation between the amounts of DTXs obtained by HPLC quantitation and the level of toxicity revealed by biological experiments can exist.

2. Experimental

2.1. Reagents and chemicals

Pure standards of destruxins (E diol [Ed], E, D, A_2, A, B_2 and B) were provided by the Comparative Pathology Research Station-INRA (Saint-Christol-lez-Alès, France). Purity of destruxins was checked as described previously [8–11]. Organic solvents, chromatographic grade (Merck, Nogent-sur-Marne, France) were filtered through an FH filter (0.5 μm) (Millipore, Molsheim, France) and 18-MΩ deionized water, obtained with a Waters Milli-Q apparatus, was filtered through an HA (Millipore) filter (0.45 μm). Other reagents, glycerol, CH_2Cl_2 and AcOEt (Prolabo, Paris, France) were of analytical grade.

2.2. Fungus and culture conditions

The isolate Ma23 is from the USDA-ARS culture collection. Primary fungal cultures were inoculated by introduction of $4 \cdot 10^5$ conidiospores per ml in a 100-ml Erlenmeyer flask containing 25 ml of Adameks medium [1]. After 5 days of incubation at 26°C on a rotary shaker at 110 rpm, 1.25 ml of this culture (blastospores) was sampled and inoculated in a 1-l Erlenmeyer flask containing 330 ml of Czapek broth with 0.5% peptone. Four fungal cultures were prepared in this way and then incubated in dark on a rotary shaker (110 rpm) at 24°C. After various incubation times (t_0, 1, 2, 3, 4, 7, 9 and 12 days), 10 ml of fungal culture were sampled from each flask and centrifuged at 8000 *g* at 10°C for 40 min. After centrifugation, mycelium (bottom) and culture medium (supernatant) were separated to be independently analysed. Culture media were also employed for bioassays on *G. mellonella* larvae.

2.3. Preparation of fungal samples

Samples of culture media, the pH of which increased gradually from 5.8 to 7.6 during incubation time, were separated in two batches. The first one (500 μl) was diluted (5×) and directly analysed by FAB–MS and HPLC, the second batch (9.5 ml) was extracted with CH_2Cl_2–AcOEt (50:50, v/v) before analysis. This step of extraction was optimized: the recoveries of DTXs (Table 1) were measured in

Table 1
Recoveries of DTXs from liquid–liquid extraction with CH_2Cl_2–AcOEt (50:50; v/v)

DTX	Recovery (%)
Ed	80±9
E	90±3
D	f(pH)[a]
A_2	91±2
A	93±2
B_2	93±3
B	96±2

[a]cf., Experimental.

quadruplicate by comparing the peak height of DTXs from extracted samples with those of crude samples (the same initial amounts of toxins were contained in both kind of samples). For D-DTX which contains a carboxylic function, recovery was dependent on pH decreasing progressively from ca. 80% at pH 4.5 to ca. 20% at pH 7.5. We have restricted the study to the 4.5–7.5 pH range in order to avoid the ring-opening of the cyclodepsipeptides by acid hydrolysis of the ester function.

Mycelia were dispersed in 10 ml of deionized water, sonicated for 3 min. After centrifugation at 5000 *g* for 15 min, they were extracted with CH_2Cl_2–AcOEt (50:50, v/v) before FAB–MS and HPLC analysis.

2.4. Evaluation of toxicity

The biological tests were performed with L6 larvae of the lepidopteran insect *Galleria mellonella L.* The toxicity was determined from triplicates of 20 larvae batch (body mass of 200 mg) by injecting 0.1 μl of culture media filtrates. The intrahemocoelic injections were performed using a Degasa micro-injector with 1-ml insulin microsyringes. The injected larvae were confined in glass jars in an incubator at 25°C and fed with honey and a wax diet. The percentage of larvae showing a complete paralysis, characteristic of the DTXs, after 30 min and 1 h was determined by counting larvae, lying on their back, which could not stand back on their feet.

2.5. Chromatographic system

A Waters HPLC system (Millipore, Saint-Quentin-Yvelines, France) including a 625 LC pump, a UV 486 absorbance detector and a 746 data integrator module was used. Injections were performed with a 91 25 080 Rheodyne valve equipped with a 5-μl loop.

The DTXs separation in fungal media was carried out with a 5-μm C_4 Nucleosil wide-pore analytical column (300 Å) (150×4.6 mm I.D.) supplied by Interchim (Montluçon, France). A C_4 Nucleosil wide-pore guard column (5 μm, 300 Å) (10×4.6 mm I.D.) from Interchim was used to protect the analytical column from adsorption of fungal components. Separations were achieved by ion-pairing in isocratic elution with water–CH_3CN–CH_3CO_2H (70:30:0.025, v/v) (pH 3.7) at a flow-rate of 0.5 ml/min. Detection wavelength was 230 nm.

Table 2
HPLC calibration data for standards of destruxins

DTX	*r*	Slope (*a*)	Intercept (*b*)
Ed	0.999	4.63×10^{-10}	7.3×10^{-6}
E	0.999	5.14×10^{-10}	-1.7×10^{-6}
D	0.999	6.18×10^{-10}	5.7×10^{-6}
A_2	0.999	4.91×10^{-10}	2.2×10^{-6}
A	0.999	4.27×10^{-10}	-1.5×10^{-6}
B_2	0.999	4.26×10^{-10}	3.1×10^{-6}
B	0.999	4.50×10^{-10}	5.1×10^{-6}

Regression equation: $C_M = aA + b$. External standard method, UV detection set at 230 nm (cf., Fig. 2).

2.6. Calibration graphs for destruxins

Quantitation of DTXs was achieved with the external standard method. Calibration graphs were obtained by linear regression analysis of the peak area (A) against concentrations (C_M) of each DTX in the range $5\cdot10^{-6}$–10^{-3} M with the equation $C_M = aA + b$. Regression data for each DTX (slope, intercept and correlation coefficient) are reported in Table 2. They were obtained from triplicate injections of five different concentrations covering the working range.

2.7. Mass spectrometry

Mass spectrometry was performed with a Nermag R-10-10-C quadrupole mass spectrometer and Spectral-30 data system (Delsi-Nermag, Poissy, France). FAB mass spectra were obtained with a M-Scan atom gun (M-Scan, Ascot, UK). Xenon atoms at 8 keV formed from a beam of 200 μA flux were employed. The FAB target consisted of a copper probe of 3 mm^2 area with an incidence angle of 45° relative to the primary beam. Sample preparation was carried out by spotting 2 or 3 μl of fungal media on the FAB probe covered with 2 μl of glycerol.

3. Results

For the study of DTXs production by the strain Ma23 we intended to transpose techniques of HPLC and FAB–MS, previously applied and developed for direct in vivo monitoring of DTXs behaviour in locust tissues [9–11], to the fungal media analysis.

3.1. HPLC

We have tested packings that were successfully used for the direct injection of insect fluid onto the column [10,11]: ISRP GFF I (Internal-Surface-Reversed-Phase-glycine-phenylalanine-phenylalanine), C_1 and C_4 wide-pore materials (300 Å). Such packings allow direct injection of biological media for drug analysis with limited risks of column clogging [12]. For fungal media, separations have been optimized by using a synthetic mixture of pure destruxins supplemented to uninfected culture media (blank). The main difficulty we had to overcome was to separate the toxins from endogenous components of this culture media. This separation appeared particularly difficult for very polar destruxins such as Ed- or D-DTXs, the latter appreciably ionized at pH>5, would elute with the culture media in the void volume of the column even under chromatographic conditions suitable for the other destruxins [water–CH_3CN (70:30, v/v)]. To succeed in distinguishing D-DTX, we have used ion-pairing chromatography with acetic acid (pH 3.7).

Finally, separation of the complex synthetic mixture (DTXs in uninfected culture medium) has been achieved with a mobile phase of 0.25% CH_3CO_2H in water–CH_3CN (70:30, v/v), either by the column-switching method involving two C_1 wide-pore columns (the first one used as a guard column) or on a C_4 wide-pore column protected by a C_4 cartridge. Concerning the ISRP GFF I packing, it appears to insufficiently retain the most polar destruxins even without any organic solvent in the mobile phase.

Once the DTXs separation was achieved, it was possible to monitor their production from Ma23 by injecting crude infected culture media at various incubation stages. Fig. 2 shows our more recent results obtained with the C_4 wide-pore packing. Thus, gradual appearence of several destruxins with time can easily be observed. We can notice first E-, A- and B-DTXs (3 days), then D-DTX (7 days) and finally Ed-, A_2 and B_2-DTXs after 12 days. Attribution of the chromatographic peaks was obtained (i) by comparison of the retention time with those of standard, (ii) by FAB–MS analysis of each collected chromatographic peak. The mass spectra showed molecular ion ($[M+H]^+$ in positive ions and $[M-H]^-$ in negative ions) as well as previously identified fragment-ions [13,14] that characterize each collected chromatographic peak.

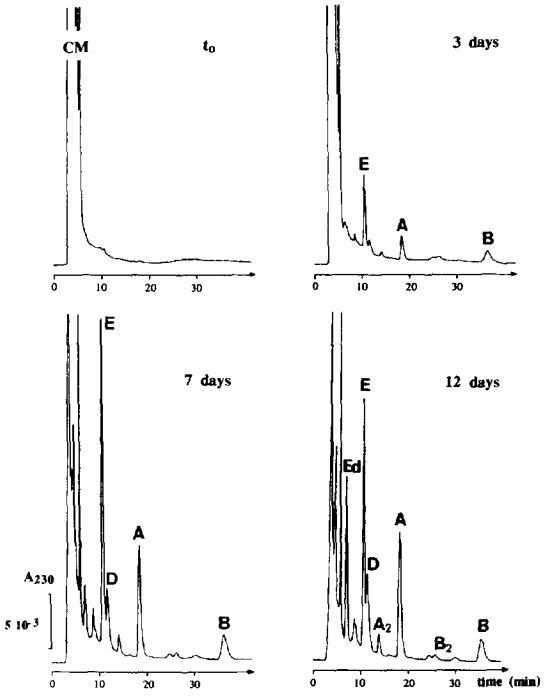

Fig. 2. Direct HPLC monitoring of DTXs production by Ma23. Chromatographic profiles of infected culture media at different incubation times. Chromatographic conditions: C_4 analytical column (Nucleosil, 5 μm, 300 Å, 150×4.6 mm I.D.) preceded by a C_4 cartridge guard column (5 μm, 300 Å, 10×3 mm I.D.). Isocratic elution with water–CH_3CN–CH_3CO_2H (70:30:0.025, v/v) (pH 3.7) at a flow-rate of 0.5 ml/min. Detection wavelength at 230 nm. Injection of 5-μl aliquots. Retention times: Ed-DTX 6.9 min, E-DTX 10.5 min, D-DTX 11.8 min, A_2-DTX 13.9 min, A-DTX 18.4 min, B_2-DTX 26.2 min, B-DTX 36.6 min. CM: Components from culture media. They were observed for blanks of culture medium.

A quantitation has been performed to estimate the toxin concentrations during incubation (Fig. 3). Thus, an increase of the amounts of all destruxins has been revealed: rapid from the second to the

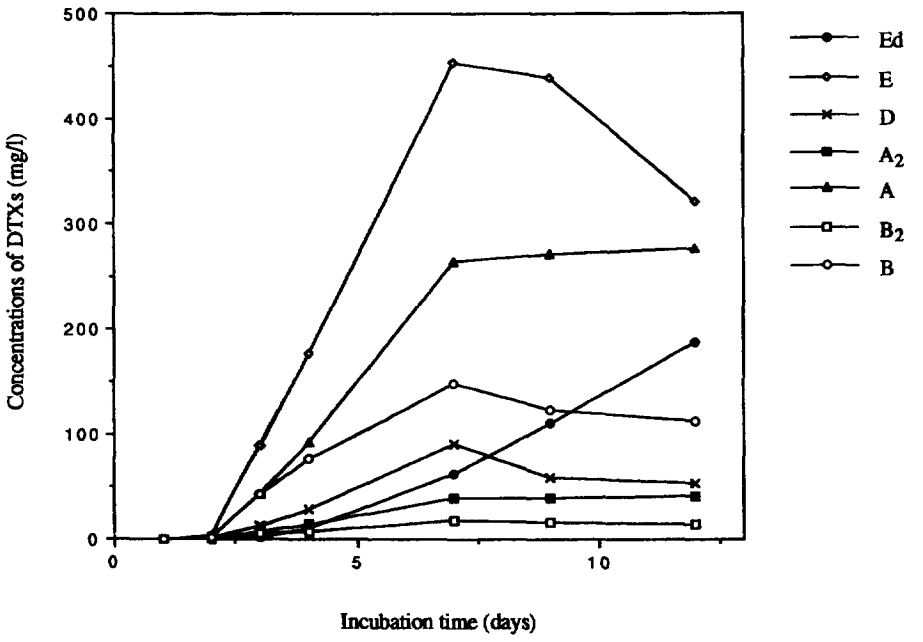

Fig. 3. Time-course of DTXs concentrations (mg/l) in infected culture media during incubation. Concentrations are average values calculated from four culture analyses. They are expressed in mg/l determined from concentrations in mol/l obtained from calibration graphs.

seventh day and then, slower. A decrease of the concentrations is also observed for some destruxins after 9 days and particularly for E-DTX.

To compare 'direct injection' with the 'classical approach', the same fungal samples have been analysed after a prior extraction. Otherwise, a comparison between culture media and mycelia has also been carried out. Injections of mycelia extracts have also been performed.

3.2. FAB-MS

Because blanks of culture media give no ionic signal in the mass range of interest, neither in positive nor in negative-FAB ionization, mass spectra of culture media and mycelia of different incubation times can be obtained from crude samples in both detection modes. Thus, as an illustration of the results, Fig. 4 shows the mass spectra of a seven days old culture medium. Protonated (positive-FAB) and deprotonated (negative-FAB) molecules of different destruxins are detected which means that Ma23 produces various components of this cyclodepsipeptidic family. In the case of positive-FAB, we have also observed destruxin adducts with sodium and potassium alkali cations present in the nutritional culture media, i.e. m/z 616 and 632 for sodium and potassium adducts of E- and/or B-DTXs. Moreover, the mass spectra show characteristic fragment-ions of each destruxin which were previously used for the sequencing of pure isolated destruxins [13,14].

3.3. Toxicity tests on G. mellonella larvae

Besides HPLC and FAB-MS analysis, biological assays have been carried out by injection of filtrates of culture media in *G. mellonella* larvae. Fig. 5 shows the evolution of the pathogenic effect of Ma23 with incubation time. A tetanic paralysis in inoculated *G. mellonella* larvae appears from the third incubation day. Then, the rate of paralysis reaches 100% very rapidly and remains at this level after four days.

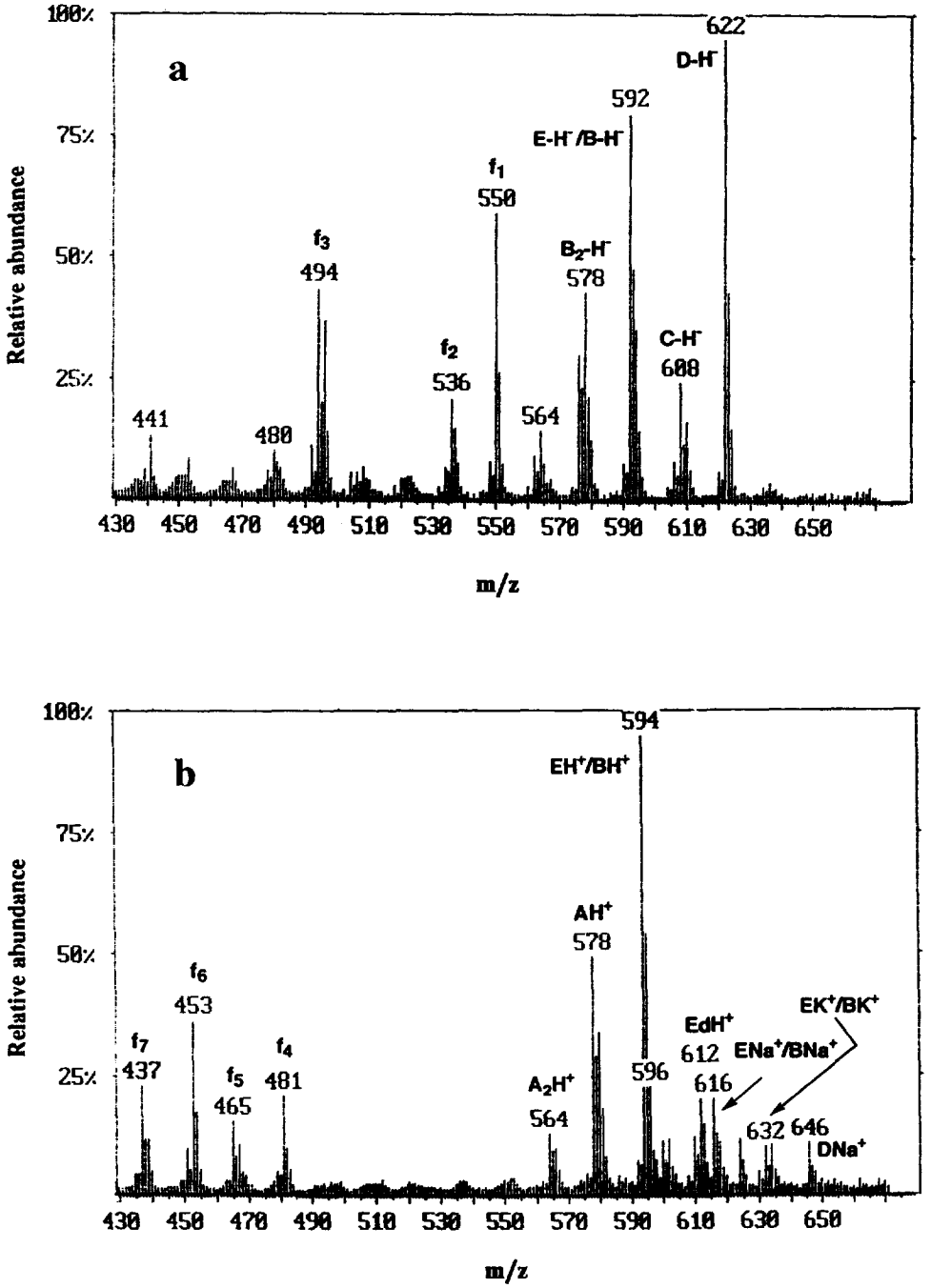

Fig. 4. FAB mass spectra of infected culture medium from Ma23 after 7 days of incubation (<Xe>, 8 keV, 200 μA). (a) Negative ions. (b) Positive ions. Characteristic fragment-ions: Negative ions, f_1 and f_3 from E-, B- and A-DTXs, f_2 from A_2- and B_2- DTXs. Positive ions, f_4 and f_6 from E- and B-DTXs, f_5 and f_7 from A-DTX.

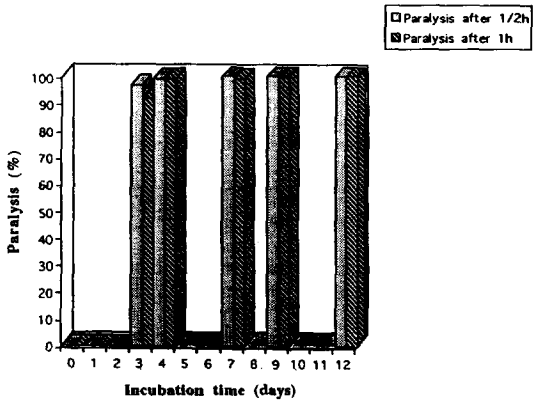

Fig. 5. Pathogenic effect of the strain Ma23. Bioassays on *Galleria mellonella* larvae. Percentages of paralysis are average obtained on three groups of 20 larvae.

4. Discussion

By using HPLC and FAB-MS, we can observe the release of DTXs into infected culture media (Fig. 2 and Fig. 4). The presence of the toxins is also detected in mycelia. In fact, culture media give chromatograms and mass spectra very similar to those of mycelia. This similarity suggests that the toxins are biosynthetized in mycelia and straight after, released in culture media. Thus, the diffusion of the destruxins is a very rapid process.

Both HPLC and FAB analysis show that numerous destruxins are produced by the fungus (Fig. 2 and Fig. 4). On the basis of HPLC retention times (comparison with the retention times of standards) and by FAB-MS analysis of each collected chromatographic peak (mass molecular and characteristic fragment-ions), attribution of twelve destruxins formed from Ma23 was performed:

(1) The presence of Ed, E, D, A_2, A, B_2 and B destruxins has been definitively established by means of pure standards,

(2) Less abundant toxins, C_2 (8.5 min), E_2 (8.5 min), C (10.5 min), Chl (11.8 min) and desMeB (24.8 min) DTXs have also be noticed.

From the HPLC quantitation (Fig. 3), an optimum stage for the destruxin production can be estimated near the seventh incubation day. Indeed, there is a general increase for all the DTXs amounts until the seventh day, then this trend is followed either by a decrease (E-, D- and B-DTXs) or by an increase more or less pronounced (A-, A_2- and Ed-DTXs).

Fig. 2 shows that concentrations are 'globally' high, particularly for the three toxins that are prominent during the whole incubation: E, A and B-DTXs. Indeed, on the seventh incubation day, they correspond to 70% of the total fungal sample in respective proportions 4, 2, 1. It must be noted that the most active toxin of the series, namely the E-DTX, is also the main product of Ma23, its amount reaching 450 mg/l at 7 days.

However, the E-DTX concentration increases up to the 7th day and then decreases noticeably. At the same time, another toxin, Ed-DTX, appears from the 4th day and its concentration raises outstandingly on the following days. Thus, we can theorize that Ed-DTX, previously described as a metabolite of E-DTX [8–10] and recently isolated [7], is not a direct product of *M. anisopliae*, but results from enzymatic hydrolysis of the epoxide function of E-DTX inside the mycelium.

4.1. Correlation with biological activity.

The results of the analysis (Fig. 3) and of quantitative bioassays (Fig. 5) concerning the rate of DTX-induced paralysis have been compared. A correlation is effectively noted between the two types of data. Thus, both methods establish that destruxins are released early in the mycological medium in detectable quantities. They also indicate an increase of DTXs concentrations between days 3 and 4. The 100% rate of paralysis, obtained on the fourth day, is caused by injection of culture medium filtrate (0.1 μl) containing 18 ng of E-DTX, 9 ng of A-DTX and 8 ng of B-DTX. Notice that the injection of filtrate of culture media sampled after 5 days has not given useful data for a comparative toxicity study because the 100% rate of paralysis has been reached as early as the fourth day. Due to the high biological activity of the filtrates of Ma23 in our optimal culture conditions, a full comparison of the toxin biosynthesis level would need additional injections of diluted filtrates.

4.2. Comparison between the 'direct' approach and the 'classical' analysis

Mass spectra and chromatograms from crude fungal samples appear to be very similar to those obtained from extracts. Indeed, the major difference lies in the fact that, in the case of HPLC, the chromatographic peak of biological medium is more important for 'direct' injection than for 'classical' analysis. Besides, in the final stage of incubation (9 and 12 days, when the pH's of the culture media are 7.45 and 7.61 respectively), we have observed a lack of D-DTX on the chromatograms of extracts, whereas chromatograms of crude samples show the presence of the toxin. Thus, there are significant losses in amounts of D-DTX after extraction due to low rates of recoveries. That result shows the major advantage of the 'direct' approach which avoids poor extraction of very polar compounds by analysing crude sample.

5. Conclusion

The use of both FAB-MS and HPLC, in sequential mode with the same sample or in the off-line mode, is a powerful and very convenient method for direct analysis of destruxin production by the fungus *M. anisopliae*. Experimental data demonstrated that the strain Ma23 is an abundant source of destruxins under optimized culture conditions. Actually, twelve different toxins of the series are formed in very significant amounts, especially for the highly toxic E-DTX. Moreover, a correlation between the destruxin production and the tetanic paralysis detected in *G. mellonella* larvae injected with culture media has been observed.

By comparing the 'classical' extractive analysis with the 'direct' HPLC and MS approach, we have simultaneously demonstrated the feasibility and the advantages of such a 'direct' approach to:

(1) Avoid selective extraction detrimental to polar molecules such as D-DTX.

(2) Allow a very simple and rapid analysis of the crude fungal samples.

In conclusion, direct analysis appears to be more suitable than the extractive procedure to study polar compounds such as destruxins. It would be very helpful in the understanding of the destruxin biosynthesis. Indeed, the presented methodology could be applied to the research of linear ring-opened destruxins which are very polar peptides, considered as potential precursors involved in the biosynthesis of the toxins.

Acknowledgments

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