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Note

Facile separation and identification of polyoxins from fermentation sources using high-performance liquid chromatography, ultraviolet spectroscopy and fast atom bombardment mass spectrometry

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The polyoxins are a group of nucleoside peptide antifungal antibiotics with sructures exemplified by structures I–IV[1] (Fig. 1). As part of a fermentation product screening program searching for new antifungal agents, procedures were needed for detecting and identifying these compounds and their analogues using minimum amounts of time and material.

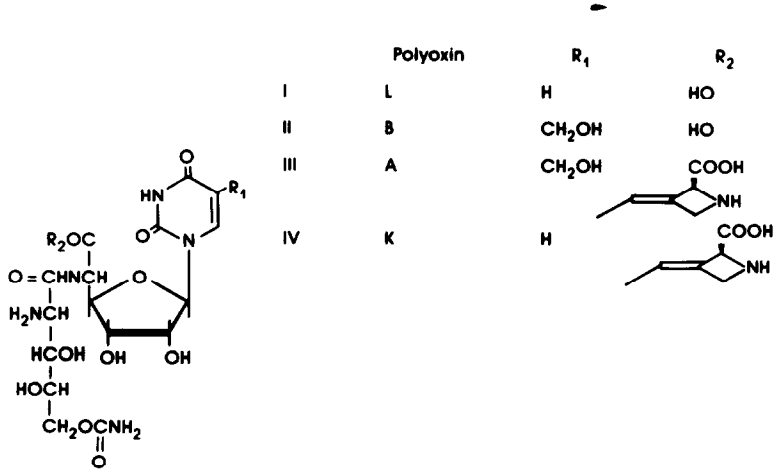

Fig. 1. Structure of polyoxins L, B, A and K.

Fast atom bombardment mass spectrometry (FAB-MS) has been found to be a powerful structural tool in determining molecular weight of highly polar non-volatile natural products[2–4] such as the polyoxins without requiring derivatization. Two recent reports[5,6] have shown that reversed-phase high-performance liquid chromatography (HPLC) can be used in the purification and quantification of polyoxins and related antibiotics. We wish to describe a facile microscale system for the separation and identification of polyoxins utilizing a combination of HPLC, UV spectroscopy and direct analysis of underivatized concentrated fractions by FAB-MS.

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EXPERIMENTAL

The analytical HPLC system used consisted of an LDC Constametric III pump, an LDC Spectromonitor III variable-wavelength UV detector operating at 254 nm, a Hewlett-Packard 3390 A integrator and a Rheodyne sample injector (Model 7120). Separations were carried out on a Whatman Partisil-10 ODS-3 reversed-phase analytical column (250 × 4.6 mm I.D.) at 1 ml/min or a Whatman Magnum 9 ODS-3 semi-preparative reversed-phase column (50 cm × 9.4 mm I.D.) at 4 ml/min. The mobile phases used were either 0.15% aqueous heptafluorobutyric acid (HFBA) containing 13% acetonitrile or 0.15% aqueous trifluoroacetic acid (TFA). Both columns were fitted with a Brownlee MPLC pre-column equipped with a 10-μm reversed-phase C_{18} cartridge. TFA and HFBA were purchased from Pierce and Kodak, respectively. UV spectra were recorded on a Beckman DU-7 spectrophotometer using the mobile phase as reference. Bioassays were performed by discing fractions against *Candida albicans* B311 grown in yeast carbon base-lysine defined medium as described by Mehta *et al.*[7].

A sample of 5 g of the crude polyoxin mixture supplied by Kaken Chemical (Honkomagome, Tokyo, Japan) was extracted with 10 ml of water. The aqueous solution was separated from the insoluble materials by centrifugation and the filtration through a Rainin Nylon-66 filter (0.45 μm).

MS was conducted in the FAB mode of operation on a VG ZAB-1F HF mass spectrometer equipped with a standard FAB ion source. The solid sample was mixed on the stainless-steel probe in a glycerol–methyl alcohol matrix and examined at room temperature. Xenon atoms (8 keV) at a discharge current of 1 mA were used to bombard the sample. Fourier transform ^1H NMR were obtained on a JEOL FX-900 instrument at 90 MHz using ^2H_2O as solvent with dioxane as internal standard.

RESULTS AND DISCUSSION

To facilitate this study, a commercial polyoxin agricultural fungicide preparation was used as a source of materials. The antibiotic complex was extracted from insoluble constituents using water and the extract analyzed by reversed-phase HPLC. Since a variety of UV-absorbing peaks were observed in the chromatogram, fractions were collected and analyzed by discing against *C. albicans* B311 to identify bioactive constituents[7]. The strain is grown in defined medium in order to enhance sensitivity for polyoxins. From a scale-up run where 25 mg of extract was injected in a mobile phase of 0.15% aqueous TFA on a Whatman M-9 ODS-3 semi-preparative column, two of the major components could be separated (Fig. 2). The major bioactive UV-absorbing peaks were collected for spectroscopic characterization. In order to simulate analyses amenable to the microscale quantities of products anticipated in fermentation broth screening, the isolates were characterized by UV spectroscopy and FAB-MS (Table I). Component I has a UV spectrum with a maximum at 259 nm. This wavelength is compatible with the presence of an unsubstituted uracil chromophore[1]. Its FAB-MS spectrum displayed a peak at m/z 478 in the positive mode and m/z 476 in the negative mode, indicating a molecular weight of 477. These data are consistent with this component being polyoxin L (Fig. 1). Although confir-

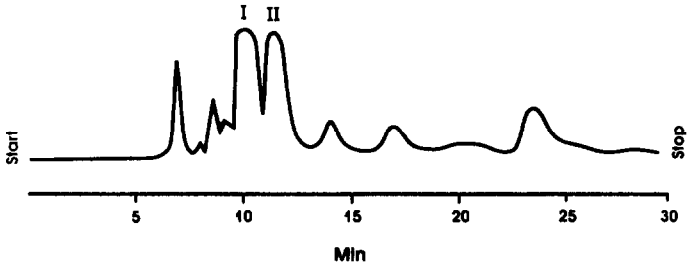

Fig. 2. Separation of polyoxins L (I) and B (II) on a Whatman Magnum 9 ODS-3 semi-preparative column (50 cm × 9.4 mm I.D.) using a mobile phase of 0.15% aqueous TFA at a flow-rate of 4 ml/min, an injection volume of 500 μl (25 mg) of the sample and UV detection set at 254 nm.

TABLE I

UV AND MOLECULAR WEIGHT DATA FOR POLYOXINS

Polyoxin	*Molecular formula (from ref. 1)*	*FAB-MS*		*Wavelength for maximum UV absorption (nm)*	
		Positive mode	*Negative mode*	*Ref. 1*	*Experimental*
L (I)	$C_{16}H_{23}N_5O_{12}$	478	476	259.0	259.0
B (II)	$C_{17}H_{25}N_5O_{13}$	508	507	262.0	262.3
A (III)	$C_{23}H_{32}N_6O_{14}$	617	615	262.0	262.3
K (IV)	$C_{23}H_{30}N_6O_{13}$	587	585	259.0	259.0

mation of this structure was not possible because of the lack of a reference standard, additional supportive data were obtained in this case by ^{1}H NMR. The ^{1}H NMR spectrum is consistent with the structure and displayed a pair of doublets [δ 5.68(d) and δ 7.35(d), J = 8 Hz] characteristic of an unsubstituted uracil vinyl group[8]. The second component was shown to be polyoxin B by analogous reasoning using UV and FAB-MS arguments and confirmed again by ^{1}H NMR spectroscopy[8]. Using an analogous procedure with a mobile phase of 0.15% HFBA containing 13% acetonitrile, two other major components were isolated (Fig. 3) and identified to be polyoxins A (III) and K (IV). These two polyoxins differ from L and B in the presence of a relatively lipophilic azetidine ring containing an exocyclic ethylidine group (see

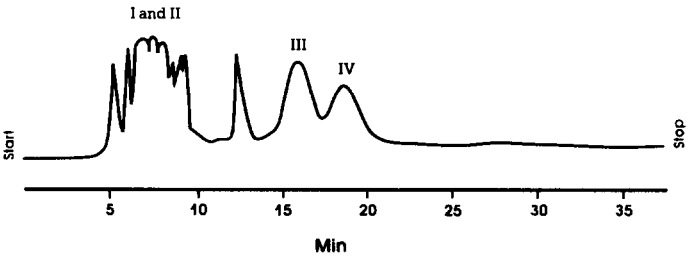

Fig. 3. Separation of polyoxins A (III) and K (IV) under the same conditions as Fig. 1 by using a mobile phase of 0.15% aqueous HFBA containing 13% acetonitrile.

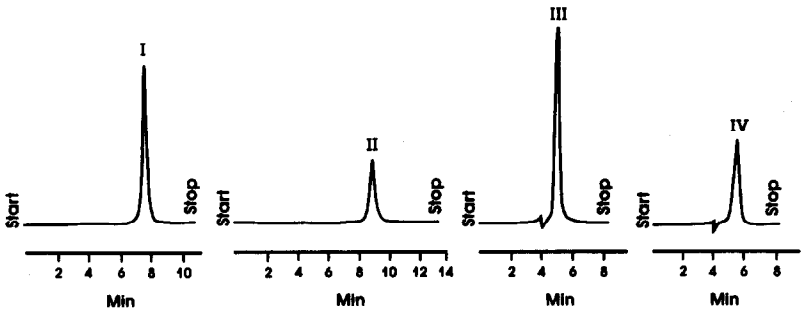

Fig. 4. Analysis of the fractions collected using a Whatman Partisil-10 ODS-3 column (250 × 4.6 mm I.D.). Polyoxins L (I) and B (II) were run in a mobile phase of 0.15% aqueous TFA while polyoxins A (III) and K (IV) were in 0.15% aqueous HFBA containing 20% acetonitrile. UV detection was set at 254 nm and the flow-rate at 1 ml/min.

Fig. 1). As such, they require an organic cosolvent for elution. Homogeneity of all preparative fractions was confirmed by reinjection onto the analytical column (Fig. 4).

Attempts to separate the four major components in a single run using a gradient program and a mobile phase consisting of either TFA or HFBA in aqueous acetonitrile all failed. Interestingly, they could be separated by a system using a solvent switch-over. Polyoxins L and B were first separated using the TFA system. The solvent was then switched to HFBA system at 7 min and polyoxins A and K were separated (Fig. 5). However, this technique could not be applied to preparative work because of slow solvent equilibrium problems.

Polyoxins L and B from the TFA system were freeze-dried directly after collection for FAB-MS (see Fig. 6). However, polyoxins A and K obtained from the HFBA system were observed to give better FAB-MS spectra upon neutralization with ammonium hydroxide before lyophilization. FAB-MS could detect as little as

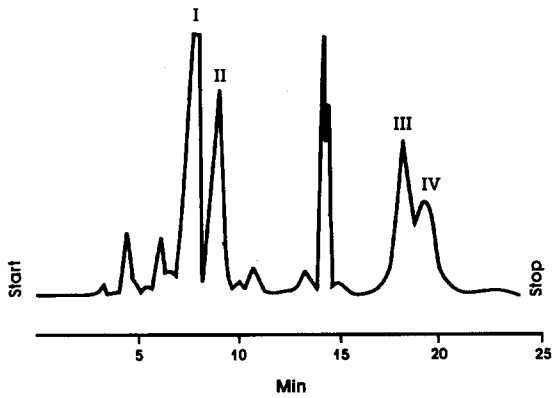

Fig. 5. Separation of polyoxins L (I), B (II), A (III) and K (IV) using a Whatman Partisil-10 ODS-3 column (250 × 4.6 mm I.D.). Solvent was switched over from 0.15% aqueous TFA to 0.15% aqueous HFBA containing 13% acetonitrile at 7 min. UV detection set at 254 nm and flow-rate at 1 ml/min.

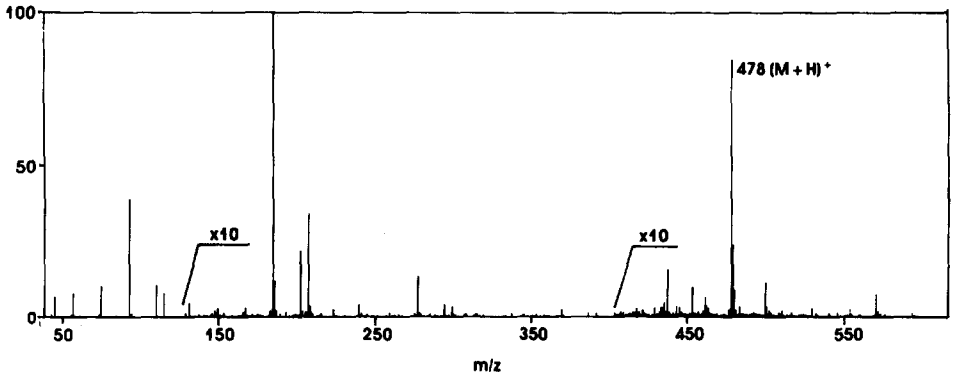

Fig. 6. FAB-MS of polyoxin L (I); positive mode.

1 μg of material isolated from the TFA system. It was observed that the presence of HFBA salt in the sample made the detection of the molecular ion five-fold less sensitive. The UV and molecular weight data for polyoxins L (I), B (II), A (III) and K (IV) are summarized in Table I.

Our results indicate that the total polyoxin content of the crude starting material upon water extraction is approximately 3% (6 mg of polyoxins L, B, A and K from 250 mg of Kaken material). The ratio of L, B, A and K in this sample were 40, 26, 20 and 15%, respectively, based on HPLC integration. It is interesting to note that our Kaken material was labeled to contain "10% polyoxin complex" bioassayed as polyoxin B while Naider's[5] sample also from a Kaken starting material was reported to contain 10% polyoxin D as the zinc salt. We could not detect polyoxin D in our extract of this complex.

In conclusion, the experimental results clearly indicate that reversed-phase HPLC can be used to isolate and purify polyoxins on a microscale and that FAB-MS is a powerful tool in obtaining molecular weight without derivatization and removal of buffer salt from HPLC fractions. To the best of our knowledge, this is the first application of FAB-MS in identifying polyoxins.

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REFERENCES

1 K. Isono and S. Suzuki, *Heterocycles*, 13 (1979) 333.
2 K. L. Rinehart, Jr., *Science (Washington, D.C.)*, 218 (1982) 254.
3 G. D. Roberts, S. A. Carr, S. Rottshaefer and P. W. Jeffs, *J. Antibiotics*, 38 (1985) 713.
4 A. K. Ganguly, B. N. Pramanik, V. M. Girijavallabhan, O. Sarre and P. L. Bartner, *J. Antibiotics*, 38 (1985) 808.
5 P. Shenbagamurthi, H. A. Smith, J. M. Becker and F. Naider, *J. Chromatogr.*, 245 (1982) 133.
6 H.-P. Fiedler, *J. Chromatogr.*, 204 (1981) 313.
7 R. J. Mehta, W. D. Kingsbury, J. Valenta and P. Actor, *Antimicrob. Agents Chemother.*, 25 (1984) 373.
8 K. Isono, K. Asahi and S. Suzuki, *J. Am. Chem. Soc.*, 91 (1969) 7490.