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Note

Purification of Polyoxin D by reversed-phase high-performance liquid chromatography

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The polyoxins are a family of peptidyl-nucleosides which are potent inhibitors of chitin synthetase from a spectrum of fungi[1,2] and arthropods[3,4]. Since chitin is a critical component of the cell-wall of these organisms the polyoxins have potential as fungicides and insecticides and are presently known to be highly toxic against both phytopathogenic fungi[5] and insects[4]. Interestingly, the polyoxins are not highly active *in vivo* against *Candida albicans* and *Saccharomyces cerevisiae* despite the fact that they inhibit chitin synthetase from both of these yeasts[5–7]. Unfortunately, few detailed studies on the polyoxins have appeared because of the limited availability of these compounds. In fact of all the polyoxins reported in the literature only one, Polyoxin D, is commercially available and its price would make any attempt at chemical modification or derivatization prohibitively expensive.

We recently embarked on an investigation aimed at gaining insights into structure–activity relationships for polyoxins in both *S. cerevisiae* and *C. albicans*. These studies necessitated 100-mg quantities of polyoxins as starting materials. As one approach to this problem we attempted to purify a crude agricultural grade Polyoxin D using high-performance liquid chromatography (HPLC). We found that highly purified samples of Polyoxin D could be obtained using a reversed-phase column. This paper presents specific details on the isolation of biologically active polyoxins by HPLC.

EXPERIMENTAL

The analytical HPLC apparatus used was a Waters chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 6000M solvent delivery system, a U6K injector and a Model 450 variable wavelength UV detector operating at 254 nm. Separations were carried out on a Waters μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D.) using either methanol–water–trifluoroacetic acid or water–trifluoroacetic acid (TFA) as the mobile phases.

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Preparative separations were accomplished on a Waters Prep LC/System 500 instrument equipped with a Prep 500-C_{18} cartridge (mean particle size 80 μm, 30 × 5.7 cm). Solvent flow-rate varied from 50 to 100 ml/min and all solvents were filtered (Millipore, 0.45 μm) before use.

The crude polyoxin was an agricultural fungicide preparation supplied by Kaken Chemical (Honkomagome, Tokyo, Japan). It is said to contain 10% Polyoxin D as the zinc salt. As supplied the material is poorly soluble in water but dissolves upon adjusting the pH to 3 using either acetic or trifluoroacetic acid. Concentrated solutions of crude Polyoxin D are dark brown and most of this color binds to the reversed-phase packing under the conditions employed for our separation.

Crude and purified polyoxins were assayed for activity using a chitin synthetase from *C. albicans* H317. A mixed membrane fraction from *C. albicans* H317 was prepared using the procedure of Chiew *et al.*[8]. Chitin synthetase was activated by incubation with trypsin and enzyme activity was measured by following the incorporation of radioactivity from UDP-[^{14}C]GlcNAc (Amersham Searle, Arlington Heights, IL, U.S.A.) into chitin. The activity of polyoxin is determined as the inhibition of chitin synthesis at various concentrations of the antibiotic.

RESULTS AND DISCUSSION

Analysis of the agricultural grade Polyoxin D on a reverse phase HPLC column revealed that rather polar solvents were required to retard the movement of the nucleoside antibiotic (Fig. 1). The peak in the crude antibiotic which corresponded to Polyoxin D was identified by comparison with a commercial sample of Polyoxin D and subsequent measurement of biological activity. The k' of the Polyoxin D

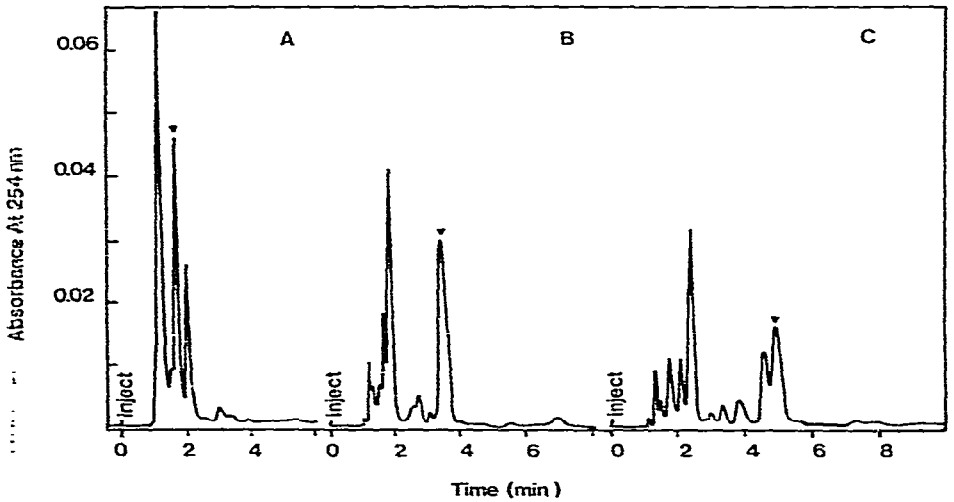

Fig. 1. Isocratic separation of crude polyoxin on a 3.9 mm × 30 cm μBondapak C_{18} reverse phase column. Mobile phases: A, methanol–water–trifluoroacetic acid (50:950:0.25); B, water–trifluoroacetic acid (99.975:0.025); C, water–trifluoroacetic acid (99.75:0.25). Flow-rate in all cases was 3 ml/min. The arrow in the figure indicates the peak corresponding to Polyoxin D. Peak I is the first major peak in panel A and elutes at 1.9 min and 2.4 min in panels B and C respectively.

peak was 1.1 using methanol–water–trifluoroacetic acid (50:950:0.25), 2.6 using water–trifluoroacetic acid (99.975:0.025) and 3.6 using water–trifluoroacetic acid (99.75:0.25). Furthermore significantly better resolution was observed using water–trifluoroacetic acid mobile phases, and with 0.25% TFA at least ten components are observed in the crude Polyoxin D (Fig. 1). According to the manufacturers specifications the use of mobile phases at low pH is expected to lead to an irreversible deactivation of the μBondapak column. In fact even at 0.25% TFA (pH = 3) we found that the column slowly loses activity over a 3–4 month period. The reported k' values are for a new column. After approximately 4 months of use the k' value in water–trifluoroacetic acid (99.975:0.025) had decreased to 1.3 and resolution was not as good as depicted in Fig. 1. We attempted to use higher TFA concentrations (0.5%, 2.0%) by adjusting the final pH to 3 with NH_4OH. No improvement in separation was observed (data not shown).

Since the HPLC analysis revealed that in addition to Polyoxin D the agricultural fungicide contained another major peak (peak I, Fig. 1), we attempted to purify both of these compounds on a preparative scale. On the basis of our preliminary studies we carried out our initial separation using 0.25% TFA. For purification 6.5 g of crude Polyoxin D were dissolved in 390 ml of 0.25% TFA. This solution was concentrated *in vacuo* to 60 ml and adjusted with neat TFA to a final concentration of 0.25%. The concentrated solution was purified in 20-ml batches (≈ 2.2 g crude

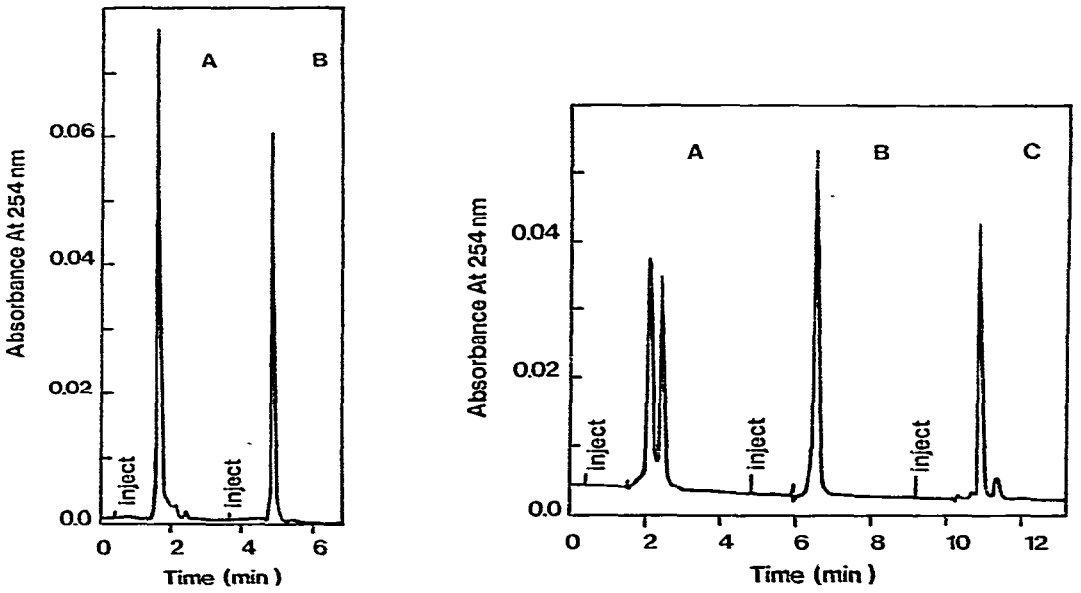

Fig. 2. Mobility of purified peak I on μBondapak C_{18} reversed-phase silica. Panel A represents pooled fractions after purification by preparative HPLC using water–trifluoroacetic acid (99.75:0.25). Panel B is peak I after a second preparative purification using methanol–water–trifluoroacetic acid (50:950:0.25) as the mobile phase. The analytical HPLC represented in the figure utilized methanol–water–trifluoroacetic acid (50:950:0.25) as the mobile phase and a flow-rate of 3 ml/min.

Fig. 3. Mobility of purified Polyoxin D on μBondapak C_{18} reversed-phase silica. Panels A and B as in Fig. 2; C is commercial Polyoxin D (Calbiochem). Other details as in Fig. 2.

polyoxin per run) using the Prep LC/System 500 and a flow-rate of 50 ml/min. Fifty-ml fractions were collected and analyzed on the analytical HPLC. Those fractions corresponding to peak I (710 mg) and Polyoxin D (590 mg) were separately pooled, evaporated *in vacuo* and freeze dried. The initial purification resulted in significant enrichment of both peak I (Fig. 2A) and Polyoxin D (Fig. 3A). Final purification was realized by changing the mobile phase to methanol–water–trifluoroacetic acid (50:950:0.25) and separately repurifying peak I and Polyoxin D. The products collected, peak I (400 mg) and Polyoxin D (230 mg), chromatograph as essentially homogeneous compounds on the analytical HPLC (Figs. 2B and 3B) using methanol–water–trifluoroacetic acid (50:950:0.25). Additional fractions containing less pure Polyoxin D (107 mg) ($\approx 80\%$ pure) were recovered.

Assay of the effectiveness of purified peak I and Polyoxin D as inhibitors of chitin synthetase from *C. albicans* H317 revealed that at 10^{-5} *M* both compounds cause on 85% decrease in the synthesis of chitin (Table I). A commercial sample of Polyoxin D (Calbiochem, La Jolla, CA, U.S.A.) had the identical activity. Ultraviolet spectroscopy gave peaks for Polyoxin D consistent with literature values for this compound[9]. The λ_{max} for peak I shows that this compound does not contain a carboxyl group on the 5′ position of the uracil ring. 300-MHz NMR spectroscopy rule out the presence of a H or CH_3 group on the 5′ position since the H in position 6′ of the uracil ring appears as a singlet at 7.37 ppm in [$^2H_6$]dimethyl sulfoxide and there is no resonance near 2 ppm corresponding to the 5′-CH_3. Based on the UV data the ring probably has a CH_2OH group in this position and by comparison of UV and optical rotation data we believe that peak I may be Polyoxin B or Polyoxin G[9]. We are currently conducting experiments designed to establish the structure of peak I and to determine whether it is one of the known polyoxins or a new compound.

TABLE I

CHEMICAL AND BIOLOGICAL PROPERTIES OF PURIFIED POLYOXINS

	Peak I	*Polyoxin D*
R_F in butanol–acetic acid–water (4:1:2)	0.11	0.06
$[\alpha]_D^{25}$	+18.5 (c, 0.2, water)	+25.6 (c, 0.18, water)
λ_{max} (nm) in 0.05 *N* HCl	263	219, 276.2
λ_{max} (nm) in 0.05 *N* NaOH	264.8	271.2
In vitro activity on chitin synthetase	85% Inhibition (10^{-5} *M*)	85% Inhibition (10^{-5} *M*)

Survey of the literature reveals few reports on HPLC of polyoxins. Recently Fiedler[10] showed that reversed-phase HPLC with ammonium formate buffer and containing heptanesulfonic acid was useful in the quantitation of the nikkomycins, a related antibiotic in fermentation broths. The results presented in this paper clearly indicate that HPLC can be used to analyze and purify polyoxins. The fact that 630 mg of purified polyoxin were recovered from 6.5 g of starting material (10% active Polyoxin D) suggests that few losses were sustained during chromatographic runs. Since the time required for all purifications is approximately 2 weeks this method can be used to prepare gram quantities of Polyoxin D. The use of TFA in the mobile phase leads to the isolation of polyoxins as their trifluoroacetate salts but does not

require a desalting step to remove buffer. Aside from the slow deactivation of the reverse columns no other deleterious effects were noted with this mobile phase.

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