SEPARATION OF DETOXIN COMPLEX AND CHARACTERIZATION OF TWO ACTIVE PRINCIPLES, DETOXIN C_1 AND D_1

Sir:

In the preceding communication[1], we have reported the production, extraction and some biological properties of the detoxin complex, obtained as the metabolites of *Streptomyces caespitosus* var. *detoxicus* 7072 GC_1.

This communication is concerned with the separation and characterization of two main principles designated detoxin C_1 and D_1.

Preliminary work by paper and thin-layer chromatography have indicated the existence of a number of similar antagonists with different specific activity. In the earlier stage of the purification, it was studied whether the minor components were real or artifacts.

We found it convenient to use buffered resin chromatography according to the procedure of MOORE *et al.*[2] for the separation of detoxin complex into eight groups according to the basicity of the components.

Detoxin complex (5.0 g, 530 u/mg) was chromatographed on a column of Dowex 50 W×2 (4×150 cm) at a flow rate of 150 ml/hour. Before use, the resin was equilibrated with 0.2 M pyridine-acetic acid buffer at pH 5.0 (161 ml of pyridine and 160 ml of acetic acid diluted to 10 liters with distilled water), and the sample dissolved in the same buffer was applied. Elution was started with the same buffer (30 liters), and then contiued with 0.3 M pyridine-acetic acid buffer at pH 5.0 (241 ml of pyridine and 200 ml of acetic acid diluted to 10 liters with distilled water). Eluate samples of 20 ml were collected mechanically and assayed for antagonism to the inhibitory action of blasticidin S against *B. cereus*. Under these conditions, eight distinct active peaks were observed in the eluate.

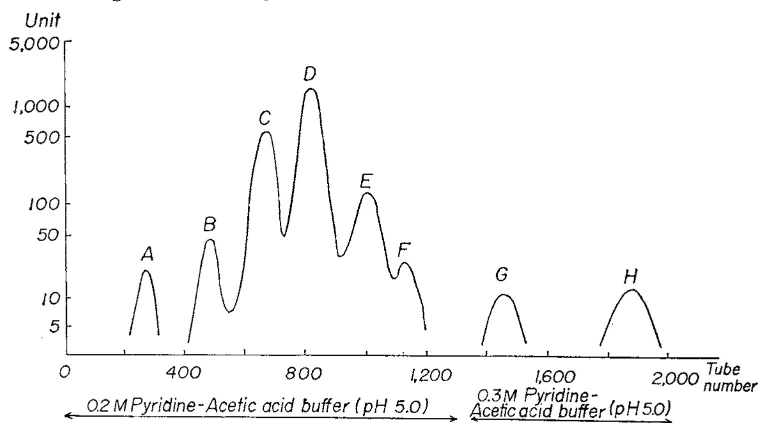
Table 1. Specific activity and yield of each group

Group	Specific activity (unit/mg)	Yield* (mg)
A	50	600
B	250	185
C	1,250	800
D	2,500	860
E	700	406
F	20	220
G	30	450
H	50	150

* Yield from 5 g detoxin complex.

Appropriate fractions under each peak were combined, concentrated under reduced pressure at 40°C and finally lyophilized to yield detoxins which were designated A, B, C, D, E, F, G, H groups in the order of elution. Each of eight groups thus obtained was examined by paper and thin-layer chromatography and the results suggested that each was still a mixture contaminated with some impurities. The results of resin column chromatography are summarized in Table 1, which suggests that groups C and D might be the main active principles of detoxin complex, because each of these two groups showed higher specific activity than others. Hence further purification was directed to these two groups.

Fig. 1. Elution pattern of resin column chromatography.



Crude detoxin C group (120 mg; 1,250 u/mg) was separated into detoxin C_1, C_2 and C_3 by partition chromatography on a silica gel column (2×20 cm,

Mallineckrodt, 100～200 mesh) using solvent system of butanol-acetic acid-water (3:1:1, v/v). Fractions of 5 ml were collected whereupon detoxin C_1 was found in tube number 18～21, detoxin C_2 in 26～30, and C_3 in 35～37. After concentration of the appropriate fractions followed by the lyophilization, detoxin C_1 (48 mg), detoxin C_2 (15 mg), and detoxin C_3 (7 mg) were obtained as amorphous powders. Detoxin C_2 and C_3 were minor components contaminated with some impurities. Detoxin C_1 was further chromatographed on Sephadex G-10 (3×120 cm) with redistillated water to afford a semicrystalline powder (18 mg; 2,200 u/mg), the purity of which was confirmed by paper and thin-layer chromatography.

Detoxin C_1 recrystallized from water gave microneedles of m.p. 142～144°C, $[\alpha]_D^{25}$= −23° (c 1, MeOH). The elementary analysis, molecular weight determination by ebullioscopic method and potentiometric titration were consistent with the molecular formula $C_{29\sim30}H_{44\sim46}O_9N_4$.

Anal. Found: C 58.59, H 7.59, O 23.43, N 9.33.
Calcd. for $C_{29}H_{44}O_9N_4$:
C 58.77, H 7.48, O 24.30, N 9.45.
Calcd. for $C_{30}H_{46}O_9N_4$:
C 59.39, H 7.64, O 23.73, N 9.24.

Detoxin C_1 is an amphoteric compound with pKa 8.0 and 3.9. UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$) 248 (3.70), 253 (3.18), 259 (2.65), 265 (2.80), 269 mμ (1.85). IR ν_{max}^{Nujol} 3300, 1740, 1670, 1580, 1250, 1200, 1170, 980 cm^{-1}.

The crude powder of detoxin D group (300 mg; 2,500 u/mg), although seemingly homogenous by paper and thin-layer chromatography, could be separated into three active components designated detoxin D_1 (150 mg), D_2 (12 mg) and D_3 (3 mg) by gel filtration on a Sephadex G-10 column (3×120 cm). Detoxin D_1 was the most important active principle of the detoxin complex with the highest specific activity (5,500 u/mg) among the detoxin components. Detoxin D_2 and D_3, the minor components of D group, were obtained in too low a yield for further characterization.

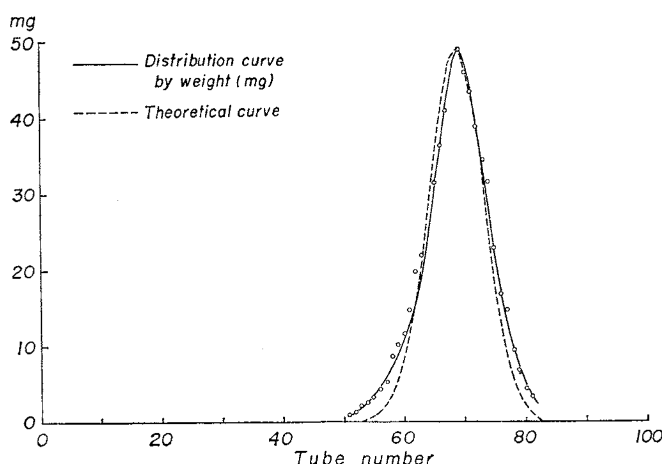


Fig. 2. Countercurrent distribution pattern of detoxin D_1.

The homogeneity of detoxin D_1 was confirmed by countercurrent distribution (solvent system: *n*-butanol-0.2 M pyridine-acetic acid buffer, at pH 5.0). The pattern is shown in Fig. 2.

Detoxin D_1, m.p. 168°, $[\alpha]_D^{25}$ −16° (c 1, MeOH), is an amphoteric compound of pKa 8.0 and 4.0 (titration equivalent 610).

Anal. Found: C 59.09, H 7.46, O 23.49, N 8.98.
Calcd. for $C_{30}H_{46}O_9N_4$:
C 59.39, H 7.64, O 23.73, N 9.24.
Calcd. for $C_{30}H_{48}O_9N_4$:
C 59.19, H 7.95, O 23.66, N 9.20.
Calcd. for $C_{32}H_{48}O_{10}N_4$:
C 59.24, H 7.46, O 24.66, N 8.64.

UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$) 253 (3.10), 258 (3.58), 265 (2.77), 268 mμ (1.85). IR ν_{max}^{Nujol} 3300, 1740, 1660, 1570, 1250, 1200, 1165, 1090, 1065, 980 cm^{-1}. Attempts to determine the precise molecular formulae of detoxin C_1 and D_1 by high resolution mass spectrometry were unsuccessful.

Further isolation and characterization of detoxin homologues, together with the structural elucidation of detoxin C_1 and D_1, are in progress.

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References

1) YONEHARA, H.; H. SETO, S. AIZAWA, T. HIDAKA, A. SHIMAZU & N. ŌTAKE: The detoxin complex, selective antagosists of blasticidin S. J. Antibiotics 21: 334~335, 1968.

2) MOORE, S.; D. H. SPACKMAN & W. H. STEIN: Chromatography of amino acids on sulfonated polystyrene resins. Anal. Chem. 30: 1185~1190, 1958.