Separation of Detoxin Complex and Characterization of Two Active Principles, Detoxin C, and D₁[†]

Noboru ÔTAKE, Katsumi KAKINUMA and Hiroshi YONEHARA

Institute of Applied Microbiology, The University of Tokyo, Bunkyo-ku, Tokyo

Received June 15, 1973

Separation of the active principles of DX.C was worked out into eight groups with the use of an ion exchange chromatography. Further purification of main components, the isolation and characterization of two main active principles were described.

In the previous report,¹⁾ the production, purification and some biological properties of DX.C were described. As it was already pointed out that, DX.C was a mixture comprised from a number of active components probably having the closely related structures. So therefore, in the earlier stage of the separation, it was noticed whether the minor components were real or artifacts. Eventually, an existence of the congeners having a different specific activity was demonstrated with combination of thin layer and paper chromatographies.

In order to the structural elucidation of these interesting antagonists, the isolation and characterization of the active principles in pure form were necessitated and were accomplished with the use of buffered ion exchange chromatography together with partition chromatography and gel filtration procedures.

This paper concerns with the details of results on the separation, characterization and properties of the active principles.

MATERIALS AND METHODS

Materials

The sample of DX.C was supplied from the Kaken Chemical Co., Ltd. All of the reagents used in this work were commercially available.

Methods

General. All mps were uncorrected. IR spectra

were measured by JASCO IRS-1 and UV spectra by Cary Model 14 spectrophotometers. Optical rotation was determined by a Perkin-Elmer 141 Polarimeter. Countercurrent distribution was carried out with automatic distributor CDA 100 of Shibata Chemical Instruments Co., Ltd. Potentiometric titration was conducted with RAT IS recording autotitrator of Hiranuma Co., Ltd.

Quantitative assays of DX activity. The same procedure was used as described in the preceding paper.¹⁾

Resin chromatography. A column packed with Dowex 50W×2 (200~400 mesh, 5×150 cm) was equilibrated thoroughly with pyridine-acetate buffer (pH 5.2, 0.2 M with regard to pyridine). five grams of DX.C dissolved in 10 ml of the buffer solution was charged on the top of the column and was successively developed with the same buffer. Twenty milliliters aliquots of the eluate were collected mechanically and each were assayed for antagonistic activity.

Partition chromatography on silica gel columns

A. Separation of detoxin C group. To a column (2×20 cm) packed with silica gel (Mallinckrodt, 100~200 mesh) using the solvent system of *n*-butanol-acetic acid-water (3:1:1, v/v), 120 mg of detoxin C mixture in 5 ml of the same solvent was absorbed and the column was successively developed. Each 5 ml of eluate fractionated mechanically was assayed for antagonistic activity.

B. Separation of detoxin D group. A column (2.5×20 cm) of silica gel (Wako gel C-200) was packed with preliminary equilibrated lower phase of *n*-butanol-pyridine-acetate buffer system (the same composition of buffer used in resin chromatography), and was washed thoroughly with upper phase. To this column, 200 mg of detoxin D mixture was charged and was separated as described above.

Gel filtration with sephadex G-10 and LH-20. Sephadex G-10 columns (3×120 cm) packed with redistil-

[†] Studies on the Detoxin Complex, The Selective Antagonists of Blasticidin S. Part II. Part I, see Reference 1). Abbreviations: BS, blasticidin S; DX.C, detoxin complex.

led water were used for separation of detoxin C and D groups, 200~300 mg of the samples were applied in this scale and each 5 ml of the eluates were fractionated. In all runs, blue dextran was used as a marker.

Sephadex LH-20 columns (2×120 cm) packed with solvent system of methanol-acetic acid-water (50:1:49) was also used in desaltation of detoxin D₁ after the separation with partition chromatography.

Countercurrent distribution of detoxin D₁. Six hundred milligrams of detoxin D₁ was distributed with the solvent system of *n*-butanol: 0.2 M pyridine-acetic acid buffer at pH 5.0 for 100 plate transfers. The distribution curve was taken by biological assay¹⁾ of appropriate tubes.

TLC analysis. TLC analysis was conducted as described in the preceding paper.¹⁾

Potentiometric titration. Potentiometric titration and measurement of pK_a were carried out according to the procedure of Parke and Davis⁵⁾ with the use of the recording-autotitrator. In each case, about 10 mg of a sample was weighed and was dissolved in 2 ml of 0.1 N hydrochloric acid. The solution was titrated with 1 N sodium hydroxide under constant flow of nitrogen gas with agitation.

RESULTS AND DISCUSSION

Separation of DX.C by resin chromatography

In the beginning of preliminary purification, the complexity of active principles in DX.C was noticed on the basis of chromatographic data and accordingly, extensive investigations about the separation procedures were worked out.

It is usually encountered that the meta-

FIG. 1. Elution Pattern of the Resin Chromatography for the Separation of DX.C.

The peaks were obtained by bioassay of the antagonistic action against BS using *B. cereus* IAM 1729.

bolites of molds contains a number of the structural analogs in concurrence with a main product and it is true, especially in the case of peptide compounds such as actinomycins²⁾ and monamycins.³⁾

On the basis of structural informations so far obtained, we found it convenient to use a bufferized resin chromatography according to the procedure of Moore *et al.*,⁴⁾ since DX.C showed an amphoteric nature obtainable by cation exchange resin from the fermentation filtrates.

DX.C chromatographed on a column of Dowex 50W×2 showed an elution pattern as shown in Fig. 1 which indicates the presence of eight distinct peaks according to the antagonistic activity of the individual fractions.

The compounds obtained after removal of the buffer solution were designated detoxin A to H groups, respectively, in the order of elution. Each group thus obtained was examined by TLC and the results suggested that each was still a mixture contaminated with some impurities.

In order to avoid a confusion based on an intermixture each other of the neighbouring compounds, the appropriate fractions of each groups were collected in clean cut around the peaks.

The specific activity¹⁾ and yields of each groups are summarized in Table I, which suggests

TABLE I. SPECIFIC ACTIVITY AND YIELD OF DETOXIN A TO H GROUPS

Specific activity of each components were measured by bioassay of the antagonistic action against BS using *B. cereus* IAM 1729. The activity was represented by "unit" as described in the preceding paper.¹⁾

Group	Specific activity (units/mg)	Yield (mg)
A	50	600
B	250	185
C	1250	800
D	2500	860
E	700	406
F	20	220
G	30	450
H	50	150

FIG. 2. Separation of Detoxin C Group by Silicic Acid Partition Chromatography.

The active peaks were obtained by bioassay of the antagonistic action against BS using *B. cereus* IAM 1729.

that detoxin C and D groups might be the main active principles of DX.C because these two groups showed higher activity than others. This result coupled with the paucity of other groups, further purification was inevitably directed to detoxin C and D groups.

Separation of detoxin C group and characterization of detoxin C₁

When detoxin C (120 mg) group was subjected to the partition chromatography on silica gel, it could be separated into three components designated detoxin C₁ (60 mg), C₂ (3 mg) and C₃ (2 mg), respectively in the order of elution. It is clear as shown in Fig. 2, detoxin C₁ is a main and the other two are minor components still contaminated with some impurities.

The *R_f* values on TLC of detoxin C₁ (0.52), C₂ (0.48) and C₃ (0.44) were determined.

Detoxin C₁ was further purified by percholation of a column of Sephadex G-10 to afford a semicrystalline powder (40 mg) showing a single spot on TLC which was recrystallized from water to yield a microneedles with mp 142~144°C, $[a]_D^{25} -23^\circ$ ($c=1$, methanol), UV $\lambda_{\text{max}}^{\text{MeOH}}$ ($E_{1\text{cm}}^{1\%}$) 248 (3.70), 253 (3.18), 259 (2.65), 265 (2.80), 269 (1.85) nm, IR $\nu_{\text{max}}^{\text{NaJOl}}$ 3300, 1740, 1670, 1580, 1250, 1200, 1170, 980 cm^{-1}

Potentiometric titration of detoxin C₁ re-

FIG. 3. Separation of Detoxin D Group by Sephadex G 10 Gel Filtration.

The active peaks were obtained by bioassay of the antagonistic action against BS using *B. cereus* IAM 1729.

vealed its amphoteric nature of p*K_a* 8.0 and 4.0 with titration equivalent 590. This data was consistent with the molecular formula C₂₉H₄₄O₉N₄ derived from elementary analysis.

Anal. Calcd. for C₂₉H₄₄O₉N₄: C, 58.77; H, 7.48; O, 24.30; N, 9.45%.

Found: C, 58.59; H, 7.59; O, 23.43; N, 9.33%.

Because the paucity of the two minor components, detoxin C₂ and C₃, their characterization by usual procedure were resigned to date.

Separation of detoxin D group and characterization detoxin D₁

The crude powder (300 mg) of detoxin D group, although seemingly homogeneous by TLC analysis was still a mixture separable into three active peaks by gel filtration using a column of Sephadex G-10 as shown in Fig. 3.

A main product (180 mg) thus obtained by gel filtration was further purified by partition chromatography between *n*-butanol: pyridine acetate buffer on a column of silica gel in order to remove the minor contaminants. This treatment gave a satisfactory result to

FIG. 4. Countercurrent Distribution Pattern of Detoxin D₁.

●—●, experimental curve; ●—●, theoretical curve. The distribution curve was obtained by bioassay of each fractions against BS using *B. cereus* IAM 1729.

remove the analogous compounds going along with the main product hitherto.

Final purification was effected by gel filtration using a column of Sephadex LH-20 which showed a good separation of colored impurities as yet followed.

A fine crystalline powder (120 mg) thus obtained was designated detoxin D₁, as a main component of detoxin D group.

The homogeneity of detoxin D₁ was confirmed ultimately by countercurrent distribution using the solvent system of *n*-butanol: 0.2 M pyridine-acetate buffer at pH 5.0. As shown in Fig. 4, the distribution pattern of detoxin D₁ obtained by the experiment shows complete coincidence with theoretical curve.

Detoxin D₁, mp 156~158°C, $[\alpha]_D^{25} -16$ ($c=1$, methanol), UV $\lambda_{\text{max}}^{\text{MeOH}}$ ($E_{1\%}^{1\text{cm}}$) 253 (3.10), 258 (3.58), 265 (2.77), 268 (1.85) nm, IR $\lambda_{\text{max}}^{\text{KBr}}$ 3400~3500, 2850 1745, 1740, 1650, 1580, 1240 cm^{-1} , is an amphoteric compound with pK_a 4.0 and 8.0 (titration equivalent 570). The molecular formula C₂₈H₄₁N₃O₈ was assigned on the basis of elementary analysis. Anal. Calcd. for C₂₈H₄₁N₃O₈: C, 61.41; H, 7.55; N, 7.67; O, 23.37%. Found: C, 61.30;

H, 7.93; N, 7.62; O, 23.78%.

It is noteworthy that detoxin D group, although it can be obtained in a reasonable yield than other groups, is a mixture of at least five active components and an isolation of detoxin D₁ in a pure form is achieved hardly by a combination of tedious chromatographic treatments. As a result of a complicated isolation procedure, another minor components of detoxin D group were inevitably left out during the separations.

With regard to these minor components of detoxin D group, it will be discussed in the following report⁷⁾ about their separation and structural elucidation by GCMS.

Clearly, detoxin D₁ is a real active principle because it has a highest specific activity of 5500 units/mg and shows ten times as active as DX.C.

The chemical studies of detoxin D₁ will be reported in the following paper.

Acknowledgement. The authors are grateful to The Kaken Chemical Co., Ltd. for the supply of DX.C. This work was supported in part by a grant of the Ministry of Education, the Government of Japan.

REFERENCES

- 1) H. Yonehara, H. Seto, A. Shimazu, S. Aizawa, T. Hidaka, K. Kakinuma and N. Ôtake, *Agr. Biol. Chem.*, the preceding paper.
- 2) S. A. Waksman and H. B. Woodruff, *Proc. Soc. Exp. Biol. Med.*, **45**, 609 (1940).
- 3) K. Bevan, J. S. Davis, C. H. Hassall, R. B. Morton and D. A. Phillips, *J. Chem. Soc. (C)*, **1971**, 514, 522, 526.
- 4) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
- 5) T. V. Parke and W. W. Davis, *ibid.*, **26**, 642 (1954).
- 6) N. Ôtake, K. Kakinuma and H. Yonehara, *J. Antibiot.*, **XXI** 371 (1968).
- 7) N. Ôtake, K. Furihata, K. Kakinuma and H. Yonehara; presented at the Annual Meeting of Agricultural Chemical Society of Japan, Tokyo, April 3, 1973, will be published.