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Identification of Actinomycins by High Performance Liquid Chromatography and Fast Atom Bombardment Mass Spectrometry

Seong-eun Cho[1], Yang Mo Goo[1] and Kyoung-Ja Kim[2]

[1]Department of Pharmarcy, Seoul National University, Seoul 151-742, Korea
[2]Department of Genetics, Soonchunhyang University, Chungnam 336-600, Korea

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An actinomycin complex isolated from culture broth of a soil microorganism, SNUS 9305-011 has been examined by High performance liquid chromatography (HPLC). From the analysis of the fractions obtained by column chromatography of the ethyl acetate extract, three actinomycin components are confirmed. The HPLC analysis is carried out with a CN-bonded nucleosil column. Comparison of the retention times of the components with those of actinomycin D, C complex, $X_{0\beta}$, and V suggests that they are different actinomycins. FAB mass spectra of the components also shows different molecular ions from those of standards and other reported actinomycins. The present work has demonstrated that actinomycin components can be separated by a CN-bonded HPLC column, and that comparison of their HPLC chromatograms with authentic samples and information on their molecular ions can be successfully employed for identification of actinomycins.

Key words: Resolution; HPLC; C10-CN column

INTRODUCTION

We have been involved in pursuing new bioactive compounds from soil microorganisms. In the course of screening of new enzyme inhibitors, especially against adenosine deaminase, which has been found to be produced in unusually large amounts and to have high activity in many cancer patients (Smith and Harrap, 1975), we have encountered a soil microorganism which produces inhibitors against the enzyme. Further studies on this soil microorganism have indicated that the organism is a *Streptomyces* spp. (Kim and Park, 1994) and it produces an actinomycin complex. During purification of the active components in the culture broth of the organism, we needed to verify whether the components of the actinomycin complex are new ones. Thus, we separated the components by high performance liquid chromatography (HPLC) and compared them with authentic actinomycins. Also, we compared their molecular ions by fast atom bombardemt (FAB) mass spectrometry technique. From these studies, the actinomycin components extracted from the culture-broth of a microoganism isolated from a soil are different from actinomyin D, $X_{0\beta}$, and V, and from the actinomycin C complex. In the present paper, we wish to report the identification of actinomycins by HPLC with information on the molecular ions. The approach will be useful for those who are searching for new bioactive compounds.

Correspondence to: Kyoung-Ja Kim, Department of Genetics, Soonchunhyang University, Chungnam 336-600, Korea

MATERIALS AND METHODS

Materials

Actinomycin D, actinomycin C complex(C_1, C_2 and C_3), actinomycin $X_{0\beta}$ and actinomycin V were purchased from Sigma.

Isolation of Actinomycin Complexes from Culture Broth of SNUS 9305-011

The *Streptomyces* spp., SNUS 9305-011, isolated from a soil sample, was grown in a medium consisting of glycerol 2.0%, NaCl 0.3%, and soy bean flour 2.0% at 28℃ for 3 days. The fermentation broth was centrifuged at 1500 rpm and the supernatant was extracted with ethyl acetate. The residue obtained after evaporation of the extract was adsorbed on silica gel and load-

ed on the top of a column packed with silica gel. The column was developed with ethylacetate-hexane to give antimicrobial fractions CSE-1-133-23, CSE-1-304-5 and CSE-1-304-6.

Bioassay

The actinomycins were assayed by a paper disk diffusin method against *Mycobacterium smegatis*. The antimicrobial assay paper disks (6 mm in diameter) were wetted with fractions (30 μl) and put on agar plates seeded with the organism. After incubation of the plate for 16 hours, the diameter of the inhibition zone was read.

HPLC Analysis

HPLC analysis was carried out with a BioRad softstart HPLC pump equipped with a Rainin UV-1 model variable wave length absorbance detector and a Hwelett Packard integrator. Signals were detected at 430 nm. The HPLC column, a CN-bonded silica gel packed column (250×4 mm, Nucleosil C10-CN) was purchased from Macherey & Nagel Co. HPLC anaysis was done with 5.0 μl of ethyl acetate solution of samples. Ethyl acetate-hexane (4:6) was passed through the column at a flow rate of 1.20 ml/minute.

RESULTS AND DISCUSSION

Fermentation of the adenosine deaminase-inhibitor producing organism, SNUS9305-011 produced yellow colored materials in broth which were extracted with ethyl acetate. The compounds which inhibit adenosine deaminase show characteristic absorbance bands around 430 nm with other abosorbance bands in the UV region. Searching for compounds showing the absorbance bands around 430 nm in a computer data base for antibiotics and other bioactive compounds (about 12000 compounds are searched; the data base was compiled at Seoul National University and the literature is covered up to 1993) revealed that the chromophores are very much characteristic of the non-peptide moiety, 2-amino-4,6-dimethyl-3-phenoxazome-1,9-dicarboxylic acid, existing in all actinomycins. Actinomycins are usually found in nature as a complex, a mixture of closely related chromopeptides, which differ only in limited areas of their peptide moieties. Many actinomycins have been isolated from soil microorganisms and many analogues have been biosynthetically produced by feeding specific amino acid analogues to the fermentation broth. Some structures of actinomycins are shown in Fig. 1 and Table I.

To investigate further whether the actinomycin complex isolated from the fermentation broth of SNUS 9305-011 is composed of structurally new actinomycins or not, it is necessary to analyze the components. Chromatographic procedures for the separation and identification of actinomycins have been reviewed (Mauger and Katz, 1978). Since HPLC techniques are

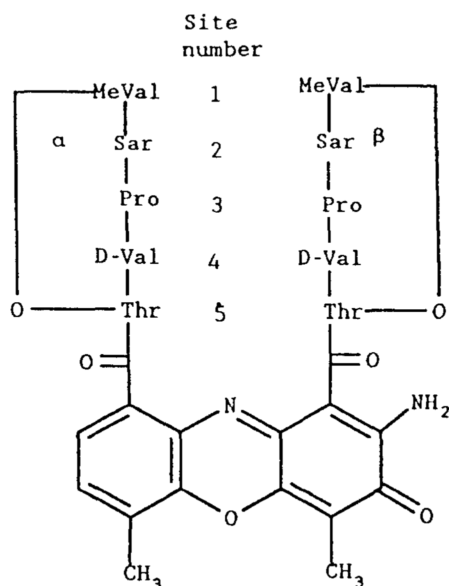

Fig. 1. Structures of congeners are defined in relation to actinomycin D by amino acid replacement according to site number.

Table I. The amino acid and its site for the replacement of the amino acid existing in actinomycin D (Fig. 1), and the retention times of actinomycins in minutes observed when they are analyzed by HPLC using a C_{10}-CN column (solvent: ethyl acetate-hexane, 2: 3, flow rate: 1.20 ml/min)

Actinomycins	Synonyms	Structure*	Mol. ion	Retention time
D	IV, C_1, X_1	–	1255	18.8 min
X_{0b}	I	3'-HyPro	1271	39.4
V	X_2	3'-(4-ketoPro)	1269	21.5
C complex				
C_1				18.8
C_2	VI	4'-D-alle	1269	16.2
C_3	VII	4,4'-D-alle	1283	13.2
CSE-1-133-23				
				19.9
			1269	22.1
CSE-1-304-5				
				19.9
			1269	22.1
CSE-1-304-6				
				19.9
			1269	22.1
			1271	26.9

*N and N' represent the amino acid site shown in Fig. 1 in α- and β-peptidechain, respectively. HyPro, ketopro, and alle stand for 4-hydroxyproline, 4-ketoproline, and allo-isoleucine, respectively.

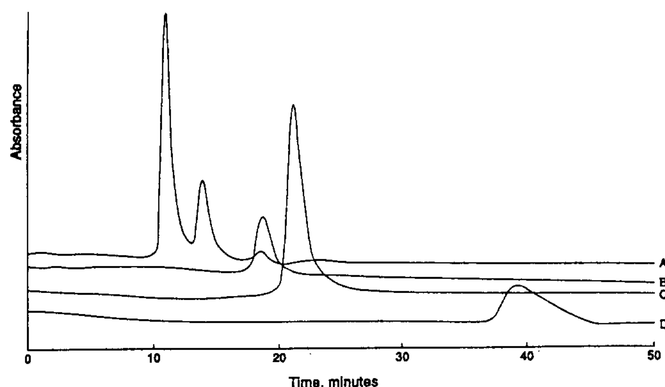

Fig. 2. HPLC chromatograms of actinomycin standards obtained at 430 nm with a Nucleosil C10-CN column (250×4 mm). Ethyl acetate-hexane (2: 3) was passed through the column at the flow rate, 1.2 ml/min a: actinomycin D, b: actinomycin C complex c: actinomycin I, d: Actinomycin $X_{0\beta}$.

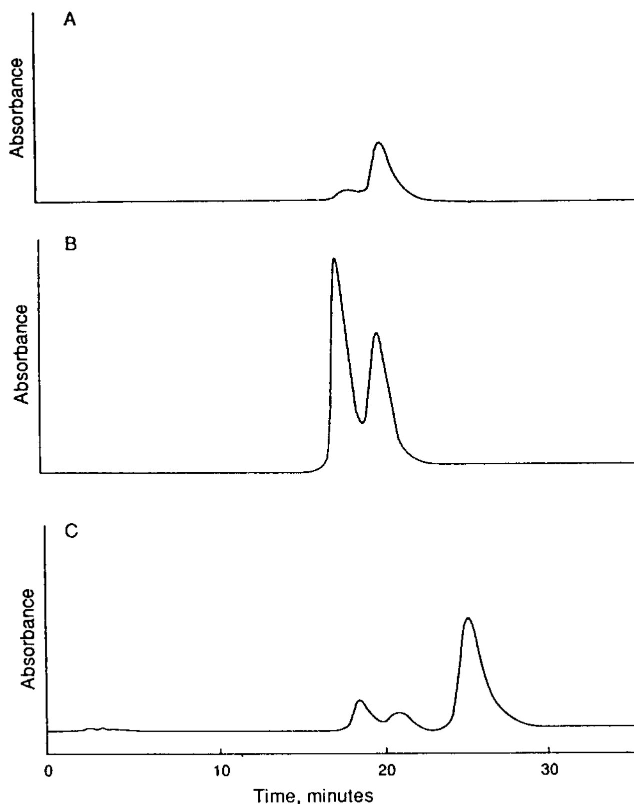

Fig. 3. HPLC chromatograms of actinomycin complexes isolated from the SNUS 9305-011 culture broth. Chromatograms were obtained under the same conditions as shown in Fig. 2. a: CSE-I-133-23, b: CSE-I-304-5, c: CSE-I-304-6.

relatively simple and rapid for quantitation and identification of bioactive compounds, it has been employed for analysis of actinomycins. The actinomycin C complex and a biosynthetic mixture of actinmycin D and congeners containing *cis*-4-chloroproline in place of one or both prolines in the α- and β-peptide chains in actinomycin molecules have been separated analytically by HPLC using a reverse-phase (C_{18}) column (Rzeszotarski and Mauger, 1973). Various other naturally occurring actinmycins and anlogues produced by direct biosynthesis have been chromatographed on both normal-phase and reverse-phase silica gel columns and their elution characteristics are compared (Mauger and Stuart, 1990). In the present study, we also employ HPLC technique to verify that the components of the actinomycin complex isolated from the culture broth of SNUS 9305-011 are structurally new ones.

Three actinomycins D, $X_{0\beta}$ and V, and actinomycin C complex comprising C_1, C_2 and C_3 which were purchased from Sigma Co. were employed as standards to give the chromatograms shown Fig. 2. The three components, C_1, C_2, and C_3 in the actinomycin C complex are resolved very nicely to give retention times of 18.8, 16.2, and 13.2 minutes, respectively (Table I). Actinomycins D, $X_{0\beta}$, and V were observed at retention times of 18.8, 39.4, and 21.5 minutes, respectively. Analysis of three fractions obtained from the column chromatography of the ethyl acetate extract of the fermentation broth of SNUS 9305-011 has shown that the isolated actinomycin complex is composed of three components. They show retention times of 19.9, 22.1, and 26.9 minutes, respectively. Retention times and chromatograms of CSE-1-133-23, CSE-1-304-5 and CSE-1-304-6 are given in Table I and Fig. 3. The retention times of the isolated actinomycin complex are apparently different from those of standard actinomycins (Table I). Fractions CSE-1-133-23 and CSE-1-304-5 give two peaks at 19.9 and 22.1 minutes, the relative amounts of which change as the collected fractions change during the column chromatography. The fraction, CSE-1-304-6 shows a third component as the major fraction with the two components observed in the previous fractions. The fractions have been further chromatographed on a column packed with silica gel to isolate individual components.

Fast atom bombardment mass spectrometry analysis indicates that two of the components have molecular ions of m/z 1269 and m/z 1271. Among naturally occuring actinomycins, actinomycin C_2, and C_{2a} are expected to give a molecular ion of m/z 1269. The expected molecular ions for actinomycin $X_{0\beta}$ and X_2 are m/z 1271 and 1269, respectively. As summarized in Table 1, the actinomycins isolated from SNUS 9305-011 have different retention times or different molecular ions from actinomycin standards. These observations suggest that the actinomycin complex isolated in the present study are composed of three new actinomycins. Currently, their structures are under invesitigation.

In this study, we have demonstrated that HPLC can

be successfully employed for the resolutof high molec weight actinomycins (ca. 1270). Authentic actinomycins as well as those obtained from a microorganism isolated from soil have been resolved very clearly. Since actinomycins are big molecules having minor differences in their peptide moieties, and since their HPLC peaks are not very sharp to distinguish some actinomycins, it is necessary to check their molecular ions to prove their identity. Thus, we have demonstrated that HPLC and FAB mass spectrometry techniques can be successfully applied to distinguish different actinomycins. The present approach will be one of the methods to be adapted by those who are faced with the question of whether a newly isolated compound is a new ones or not in the existing family of bioactive compounds.

ACKNOWLEDGMENT

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