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Enduracidin, a New Antibiotic. VI.[1] Separation and Determination of Enduracidins A and B by Column Chromatography

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Enduracidin, a depsipeptide antibiotic, was separated into A and B by column chromatography. The amino acid compositions of A and B were found to be the same. This method also can be used for the quantitative separatory analysis of enduracidin A and B.

Enduracidin was obtained from the mycelium of *Streptomyces fungicidicus* No. B-5477[3] and was found to be a novel peptide antibiotic containing chlorine in the molecule.[4] The antibiotic was reported to be composed of aspartic acid, threonine and/or allothreonine, serine, glycine, alanine, citrurine, ornithine, 4-hydroxyphenylglycine (K_1),[4] 3,5-dichloro-4-hydroxyphenylglycine (K_2),[4,5] α-(*S*)-amino-β-4(*R*)-(2-iminoimidazolidinyl)-propionic acid (Y_1) and α-(*R*)-amino-β-4(*R*)-(2-iminoimidazolidinyl)-propionic acid (Y_2) and a lipophilic substance.[1] This antibiotic has hitherto been considered as a single compound from the data of paper chromatography and countercurrent distribution.[4]

It is generally known that most of the antibiotics produced by *Streptomyces* contain several homologues. This paper deals with the separation of enduracidin into two components by column chromatography using an adsorption resin column (Amberlite XAD-2), linear gradient elution and automatic monitoring of ultraviolet (UV) absorption. The separated components were named enduracidin A (I) and B (II) respectively.

The amino acid compositions of I and II were found to be the same. This method can be used for the quantitative analysis of I and II in a mixture.

Experimental

1) **Separation Method**——The small particles of Amberlite XAD-2 resin were prepared as follows; the commercially obtained resin (20—50 mesh) was ground and the particles between 100 and 200 mesh were collected by sieving and repeated decantation. The resin was washed in succession with 1N NaOH, water,

1) Part V: S. Horii and Y. Kameda, *J. Antibiotics*, **21**, 665 (1968).
2) Location: *Juso, Higashiyodogawa-ku, Osaka.*
3) E. Higashide, K. Hatano, and M. Shibata, *J. Antibiotics*, **21**, 126 (1968).
4) M. Asai, M. Muroi, N. Sugita, H. Kawashima, K. Mizuno, and A. Miyake, *J. Antibiotics*, **21**, 138 (1968).
5) K. Kamiya, M. Nishikawa, H. Matsumaru, M. Asai, and K. Mizuno, *Chem. Pharm. Bull.* (Tokyo), **16**, 2303 (1968).

1N HCl, ethanol (2 times), ether, petroleum ether, ethanol and water. Finally the resin was equilibrated by washing several times with 0.5% NaCl in 50% methanol (solvent A) and packed into the column up to a height of 16 cm (1.8×20 cm, glass column with a jacket). The column was connected to the Beckman Model-130 Spectrochrom Analyzer and warm water (30°) was circulated in the jacket. The two reservoirs connected to the gradient pump of the Spectrochrom Analyzer were filled, one with solvent A, the other with 3/500 N HCl in 50% methanol (solvent B). The solvent A was pumped into the column at a flow rate of 60 ml/hr to complete equilibration. The sample of enduracidin (5—50 mg) was dissolved in 1—2 ml of solvent A and placed on the top of the column in the usual way, and then elution was started using the linear gradient cam and gear for 1 liter in total volume. After the measurement of the UV absorbance at 233, 253, and 272 nm, conductivity and pH of the eluate, it was fractionated every 10 min by a fraction collector at 2—5°.

2) **Quantitative Analysis**——For quantitative analysis, a smaller column (0.9×25 cm) packed with smaller particles (200—400 mesh) of the resin was used, and the column temperature was raised to 40°. In this case, a nine-chambered varigrad and Accu-flow pump (Beckman) were used for elution. The solvent placed in each chamber of the varigrad is shown in Table I. The flow rate was adjusted to 100 ml/hr. The size of the samples used for quantitative analysis was 0.5 to 2 mg and the quantities of I and II were calculated by the Height-Width method from the individual peaks.

Table I. Preparation of the Varigrad for the Chromatography

Chamber No.	Solvent A (ml)	Solvent B (ml)
1	48	2
2	50	0
3	30	20
4	26	24
5	10	40
6	42	8
7	0	50
8	0	50
9	0	50

3) **Assay of Antimicrobial Activity**——The fraction obtained by the separation of 50 mg of the sample using a large column (1.8×20 cm) were also subjected to the assay of antimicrobial activity by the paper disc method. The paper disc (Toyo Roshi Kaisha, Ltd., 8 mm in diameter) was dipped in the fractionated eluate, dried on a filter paper, and then placed on an agar plate medium suspending *Sarcina variabilis*. After standing for 1 hr at 2°, the medium was incubated at 37° for 15 hr. The comparative antimicrobial activity was represented by the content of I or II (μg/ml) which was calculated from the diameter of the zone of inhibition (the diameter of the paper disc, 8 mm, was subtracted from the apparent diameter), and it is shown in the elution pattern (Fig. 2).

4) **Analysis of Amino Acid Composition**——Enduracidin (20 mg) was separated into I and II by the above method. Several fractions near the top of each peak were pooled, concentrated and hydrolyzed with 6N HCl in an ampoule at 105°. The hydrolyzate was evaporated *in vacuo* to dryness on a boiling water bath and dissolved in 2.5 ml of citrate buffer, pH 2.2, then analyzed by an amino acid analyzer (Hitachi KLA-3B).

Result and Discussion

The separation of enduracidin cannot be performed by the usual ion-exchange chromatography, because it is comparatively insoluble in water. Our purpose in this experiment was separation of the homologues which have closely similar characters. In such a case, many workers have used adsorption chromatography for the separation. Recently, a unique adsorption resin, *i.e.* Amberlite XAD-2, has been developed and sold by the Rhom and Haas Co. In our preliminary experiment, it was found that enduracidin was adsorbed on the resin from a solution of solvent A and desorbed by solvent B. Therefore the chromatography of enduracidin was carried out on an Amberlite XAD-2 resin column and each component was eluted out by gradient elution using solvent A as the starting solvent and solvent B as the limiting solvent as described above.

The elution pattern obtained with a 6 mg sample is shown in Fig. 1. If the sample contained any impurities, most of them were eluted out before enduracidin A under these conditions. When the sample was increased to 50 mg, the peaks of both I and II appeared slightly earlier, but the separability was not decreased (Fig. 2). The curve of antimicrobial

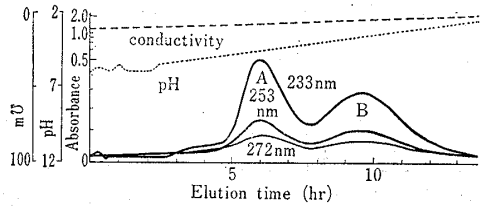

Fig. 1. Chromatographic Separation of Enduracidin A and B using a 6 mg Sample

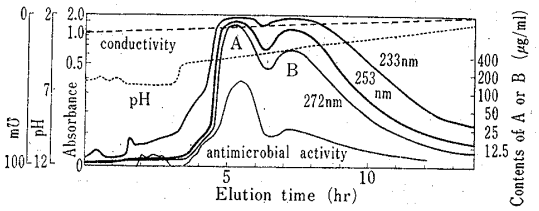

Fig. 2. Chromatographic Separation of Enduracidin A and B using a 50 mg Sample

Table II. Amino Acid Composition of Enduracidin A and B (molar ratio relative to Asp=1)

Amino acid	Enduracidin A	Enduracidin B
Asp	1.0	1.0
Thr+aThr	2.89	2.92
Ser	0.89	1.00
Cit	0.47	0.48
Gly	1.03	1.07
Ala	1.00	1.04
K_1	4.09	4.29
K_2	1.13	1.12
Orn	1.44	1.53
NH_3	8.80	8.41
Y_1	1.14	1.10
Y_2	1.05	1.00

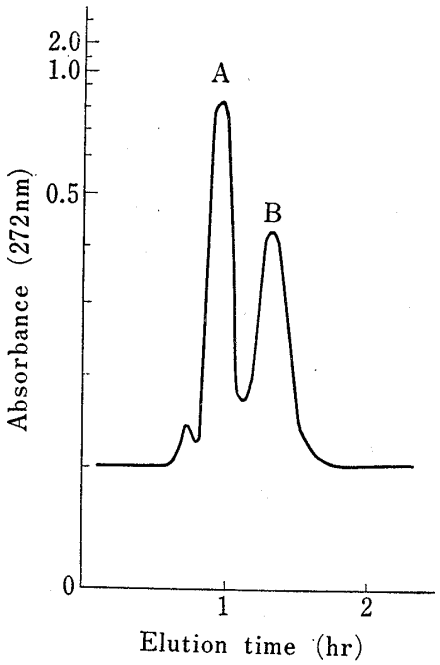

Fig. 3. Chromatogram for Quantitative Analysis obtained using a Smaller Column

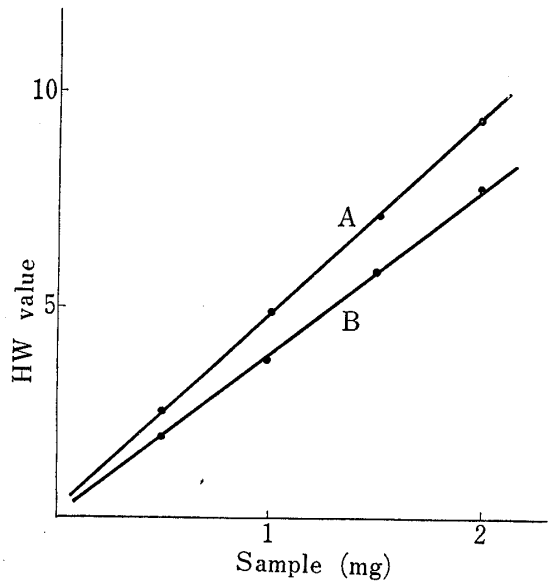

Fig. 4. Calibration Curves of Enduracidin A and B Calculated from the Area of the Peaks in the Chromatogram

activity determined by the paper disc method is also shown in Fig. 2. When separated I and II were rechromatographed individually, the peaks of I and II appeared at the same position as that in the first chromatography. This shows that these compounds were not decomposed by the adsorption resin chromatography. The amino acid compositions of these compounds are compared in Table II. The amino acid compositions of both I and II were almost the same. The fact that the values of citrulline were smaller than one mole may be due to its hydrolysis to ornithine since the values of ornithine were larger than one mole.

To increase the efficiency of the determination method for quantitative analysis, the method was modified as described in section 2) of the Experimental. The chromatogram obtained by this method is shown in Fig. 3. The relation between the area of each peak at 272 nm and the quantity of I or II was linear in the range of 0.5 to 2.0 mg as shown in Fig. 4.

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