SELECTIVE CLEAVAGE OF A PEPTIDE ANTIBIOTIC, COLISTIN BY COLISTINASE

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A colistin-inactivating enzyme, colistinase was produced by *Bacillus polymyxa* var. *colistinus* KOYAMA, a colistin-producing microorganism. The crude colistinase was fractionated as two components (colistinase I and II) by Sephadex G-50 gel filtration. Colistinase II was further purified and then, it showed as a single band in polyacrylamide disc gel electrophoresis. The molecular weight of colistinase II was about 20,000 by Sephadex G-100 gel filtration and the isoelectric point was at about 8.3. Colistinase II cleaved specifically between the 2,4-diaminobutyric acid of the side chain and 2,4-diaminobutyric acid adjacent in the cyclic peptide portion of colistin molecule.

A peptide antibiotic colistin (polymyxin E) produced by *Bacillus polymyxa* var. *colistinus* KOYAMA is a mixture of colistin A and B and composed of 2,4-diaminobutyric acid (DAB), threonine (Thr), leucine (Leu) and 6-methyloctanoic acid (MOA) in colistin A, or isooctanoic acid (IOA) in colistin B.

Previously, we reported a series of the studies on the biosynthesis of colistin by *Bacillus polymyxa* var. *colistinus*.[1~5] In these papers, it was reported that colistin activity was inactivated by a proteinase-like enzyme produced simultaneously in the fermented broth. The inactivated enzyme was tentatively designated 'colistinase' and partially purified and some properties of the enzyme were examined.[1]

In the present study, we investigated the selective cleavage of colistin peptide bond using colistinase further purified from the fermented broth of *Bacillus polymyxa* var. *colistinus* KOYAMA.

Materials and Methods

Chemicals

Carboxypeptidase A (PMSF treated) was purchased from Worthington Biochemical Co., a kit of calibration proteins to determine the molecular weight of proteins from Boehringer Mannheim Gmbh. and pI marker proteins from Oriental Yeast Co.

Microorganisms and cultural conditions

Bacillus polymyxa var. *colistinus* KOYAMA and *Escherichia coli* NIHJ were used throughout this study. The cultural conditions were described in the previous paper.[1]

Assay method for colistinase

Colistinase activity (colistin-inactivating activity) was microbiologically assayed using *Escherichia coli* as described previously.[1]

Protein determination

Protein concentration was determined spectrophotometrically by the absorbance value at 280 nm.

Amino acid analysis

Peptide was hydrolyzed in 6 N HCl at 110°C for 24 hours. Hydrolyzate of peptide was analyzed with the Nihondenshi amino acid analyzer type JLC-6AS.

Fatty acid analysis

The hydrolyzate was analyzed with gas chromatography, as described previously.[2]

Polyacrylamide disc gel electrophoresis

Disc electrophoresis in polyacrylamide gel was performed in Tris-glycine buffer (pH 8.3), with a constant current of 5 milliamperes per tube for 30 minutes according to the method of DAVIS.[6]

Thin-layer gel isoelectrofocussing

Gel isoelectrofocussing was performed at 4°C using on LKB multiphore apparatus and ampholine thin-layer polyacrylamide gel plate (ampholine range, pH 3.5 to 9.5) prepared as described by the manufacturer.[7] After focussing, the pH gradient was determined for each by cutting out small gel pieces and soaking them in 2 ml of H_2O. The isoelectric point was determined using the standard pI marker, for cytochrome C (from horse, pI 10.6); myoglobin (sperm whale, 8.7); myoglobin (horse, 7.6); cytochrome C_2 (*Rhodospirillum rubrum*, 6.2); cytochrome C_1 (*Rhodospirillum rubrum*, 5.6); and acetylated cytochrome C (horse, 3.9).

Peptide determination

The level of peptide was measured by the ninhydrin method of YEMM and COCKING.[8]

Determination of N-terminal amino acid

The N-terminal amino acid of the fragment was determined by the DNP method of SANGER.[9] Identification of 2-DNP-DAB and 4-DNP-DAB was performed according to the method described by CHIHARA *et al.*[10]

Determination of C-terminal amino acid

The C-terminal amino acid was determined by carboxypeptidase A method.[11] The liberated amino acids were analyzed with the amino acid analyzer.

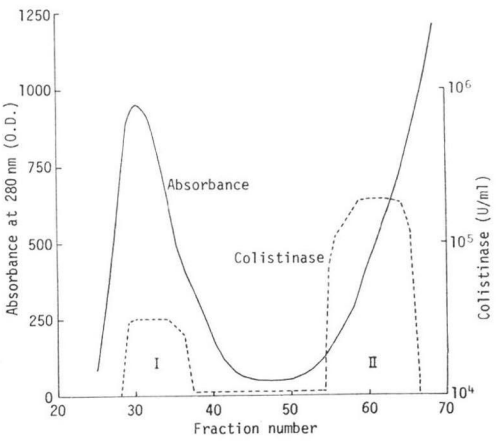
Results

Further Purification of Colistinase

All purification steps were carried out at 4°C. The culture filtrate (5 liters) was made 60% saturated with ammonium sulfate. The resulting precipitate was collected by centrifugation and dissolved in H_2O. The crude enzyme solution was applied to a Sephadex G-50 column (4.5×120 cm), the column was eluted with H_2O and 15 ml each of the fractions was collected. As shown in Fig. 1, colistinase activities were eluted as two components (colistinase I and II). Total activity of colistinase II was 98.3×10^6 units and about 3 times that of colistinase I. Colistinase II was further purified as follows: The main fractions (55~65) were combined, adjusted to pH 9.0 and applied to a column (3.0×35 cm) of DEAE-Sephadex A-50 equilibrated with 10 mM Tris-HCl buffer (pH 9.0). The column was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The fractions containing the activity were combined, dialyzed against H_2O, adjusted to pH 6.9 and applied to a hydroxylapatite column (3.0×20 cm) equilibrated with 10 mM phosphate buffer, pH 6.9. The column was eluted with a gradient of 0.01 M to 0.05 M phosphate buffer, pH 6.9. Further final purification was attempted using

Fig. 1. Gel filtration of crude enzyme preparation from culture filtrate of colistin-producing organism on Sephadex G-50.

The crude enzyme solution was applied to Sephadex G-50 column (4.5×120 cm) and 15 ml fractions were collected at the flow rate of 60 ml per hour.



Sephadex G-100. The specific activity of the fraction in the final purification step was 6.5×10^5 U/mg of protein. The purified final product migrated as a single band on polyacrylamide disc gel electrophoresis at pH 8.3. The enzyme preparation from the final Sephadex G-100 gel filtration was subjected to the following experiments throughout this study.

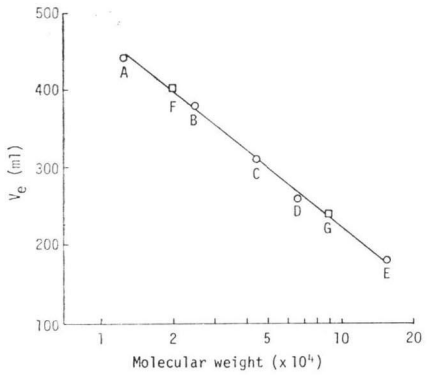
Estimation of the Molecular Weight of Colistinase II

Molecular weight was estimated according to the method of ANDREWS.[12] Each 5 mg of a standard protein and 3.5 mg of colistinase II dissolved in 3 ml of 10 mM acetate buffer (pH 5.0) were applied to a Sephadex G-100 column (2.8×120 cm) equilibrated with the same buffer and eluted with the same buffer at the flow rate of 20 ml per hour. Fig. 2 shows the plot of elution volume (Ve) through a Sephadex G-100 column against logarithms of molecular weight for the standard proteins and colistinase II. The molecular weight of colistinase II was caliculated to be about 20,000. The molecular weight of the crude colistinase I was about 88,000 by the same method. Colistinase I seems to be a tetramer, but its function is not yet clear.

Fig. 2. Determination of molecular weight of colistinase II by Sephadex G-100 gel filtration.

Ve is the elution volume of proteins.

A, cytochrome C (molecular weight 12,500); B, chymotrypsinogen A (25,000); C, Albumin from hen egg (45,000); D, Albumin from bovine serum (67,000); E, Aldolase (158,000); F, Colistinase II; G, Colistinase I.



Estimation of Isoelectric Point of Colistinase II

In isoelectric focussing on a thin-layer of polyacrylamide gel using ampholine pH range 3.5 to 9.5, colistinase II migrated as a single band with isoelectric point, 8.3, which was coincident with colistinase activity (Fig. 3).

Fig. 3. Isoelectric focusing electrophoresis of colistinase II on polyacrylamide gel plate.

A, acetylated cytochrome C (pI 3.9); B, cytochrome C_1 (5.6); C, cytochrome C_2 (6.2); D, myoglobin (7.6); E, myoglobin (8.7); F, cytochrome C (10.6); G, colistinase II.

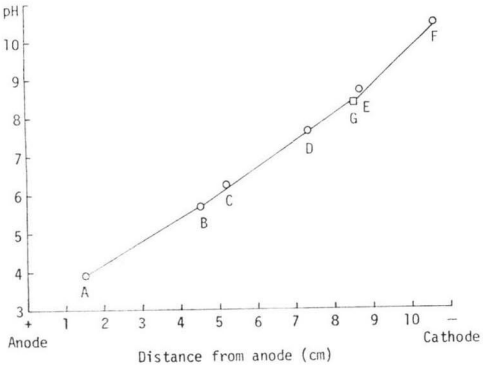
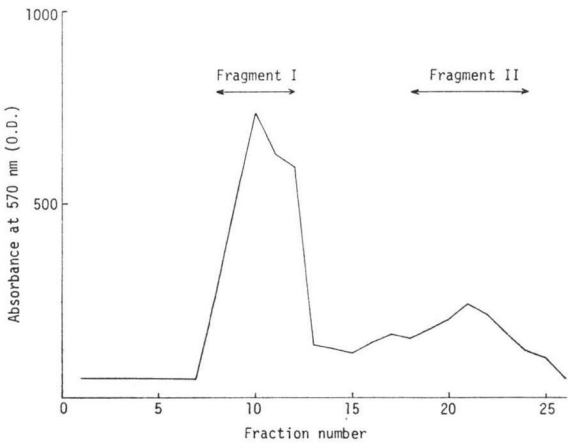


Fig. 4. Purification of colistin digested by colistinase II by an Amberlite IRC 50 column chromatography.

Colistin sulfate was cleaved by colistinase II and the resulting peptides were fractionated on a column (3×18 cm) of Amberlite IRC 50 (H^+ form) in the mixture of methanol - HCl - H_2O (45: 5: 50, vol.). The effluent was collected in 10 ml fractions with the flow rate of 30 ml per hour and 0.1 ml aliquot was assayed for the ninhydrin method.



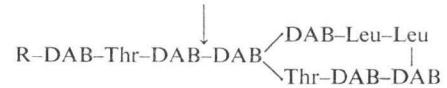
Selective Cleavage of Colistin by Colistinase II

The reaction mixture, containing 5.0 ml of colistin sulfate solution (40 mg/ml), 10 ml of colistinase II (6×10^5 U/ml as colistinase activity) and 15 ml of 100 mM borate buffer (pH 9.0) was incubated at 37°C for 3 hours. After incubation, the reaction mixture was acidified by adding diluted HCl at pH 2.0 and applied to a column (3×18 cm) of Amberlite IRC 50 (H^+ form). The column was washed with 300 ml of H_2O to exhaustively remove the ninhydrin positive substances, which was subsequently eluted with the mixture of HCl - methanol - H_2O (5: 45: 50, vol.). Ten ml fractions were collected. An aliquot of the effluent (0.1 ml) was taken for assay by the ninhydrin method. The chromatographic elution pattern obtained by monitoring with ninhydrin is shown in Fig. 4. Two main fragments were detected and designated as fragment I and II. Fragment I (fraction 8~12) gave the Rf value of 0.36 as one spot on a paper chromatogram using a solvent system of butanol - acetic acid - H_2O (4:2:1, vol.), while fragment II (fraction 18~24) had an Rf value of 0.66 in the same solvent system. Each fragment I and II fractions were combined, concentrated under the vacuum, adjusted to about pH 2.0 with Amberlite IRA 410 (OH^- form) and subsequently a pale yellow powder was obtained by lyophylization, respectively. These preparations were subjected to N-terminal, to C-terminal amino acid and to amino acid analysis as described in Methods. No free amino acids were detected when after digestion by colistinase, the reaction mixture was subjected to a paper chromatography or amino acid analyzer. An acid hydrolyzate of fragment I or II was analyzed with amino acid analyzer. The molar ratio of amino acids in fragment I was established as Thr: Leu: DAB = 1: 2: 4 and in the fragment II as Thr: DAB = 1: 2. N-Terminal amino acid residue in fragment I was DAB, while no C-terminal amino acid residue, 6-methyloctanoic and isooctanoic acids could be detected. In fragment II, DAB was identified as the C-terminal amino and residue, and moreover, 6-methyloctanoic and isooctanoic acids were detected.

The results are summarized in Table 1.

Fig. 5. The specificity of colistinase II toward a peptide antibiotic colistin.

The arrow indicates the site of action of the enzyme under the experimental condition described in Results.



colistin A: R = 6-methyloctanoic acid (MOA)
colistin B: R = isooctanoic acid (IOA)
DAB : 2,4-diaminobutyric acid

Table 1. Amino acid composition of fragments obtained from digestion of colistin by colistinase II.

Amino acid and fatty acid	Fragment (molar ratio)	
	I	II
Threonine (Thr)	1.08 (1)	1.16 (1)
Leucine (Leu)	2.0 (2)	—
2,4-Diaminobutyric acid (DAB)	3.84 (4)	2.0 (2)
N-Terminal amino acid	DAB	—
C-Terminal amino acid	—	DAB
Fatty acids	—	6-methyloctanoic and isooctanoic acids (MOA) and (IOA)
Sequence	DAB—Leu—Leu DAB Thr—DAB—DAB	MOA—DAB—Thr—DAB (IOA)

From these results, it is concluded that colistinase II from *Bacillus polymyxa* var. *colistinus* KOYAMA cleaves between the 2,4-diaminobutyric acid of the side chain and DAB in the cyclic peptide portion adjacent (DAB-DAB), as indicated in Fig. 5. Although the DAB-DAB bond in the side chain was specifically hydrolyzed by colistinase II, the other DAB-DAB bond involved in cyclic peptide portion was not split.

Discussion

As described previously, colistinase had similar properties to a serine alkaline proteinase 'nagarse' from *Bacillus subtilis*.[1] Moreover, colistinase I and II are sensitive to proteinase inhibitors such as phenylmethylsulfonyl fluoride and potato inhibitor but not to tosyl-L-lysine chloromethylketone or tosyl-L-phenylalanine chloromethylketone.[13] From these views, it is clear that colistinase is a kind of a serine alkaline proteinase. The results described in this paper show that colistinase participates in the degradation of colistin produced in the fermented broth and its inactivation is due to hydrolysis of the specific peptide bond, DAB-DAB as indicated in Fig. 5. However, further examination is necessary to see whether colistinase I cleaves the same site of colistin.

On the other hand, SUZUKI *et al.* investigated the chemical structure of colistin A according to the enzymatic method using nagarse.[14] Nagarse gave three peptides, MOA-DAB-Thr-DAB, MOA-DAB-Thr and cyclo-DAB-DAB-Leu-Leu-DAB-DAB-Thr-, and free DAB. However, the production of MOA-DAB-Thr and free DAB were due to the further hydrolyzation by nagarse for a long 72 hours.

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