KEDARCIDIN, A NEW CHROMOPROTEIN ANTITUMOR ANTIBIOTIC

II. ISOLATION, PURIFICATION AND PHYSICO-CHEMICAL PROPERTIES

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Kedarcidin, a new chromoprotein antitumor antibiotic, was isolated from the culture broth of a novel actinomycete strain L585-6 (ATCC 53650). The antibiotic was recovered from the culture filtrate by adsorption to QAE ion exchanger and purified by successive application of gel filtration and ion exchange chromatography with Sephadex G-50 and DEAE-Sephadex, respectively. Kedarcidin is an acidic complex (pI 3.65) with an apparent molecular weight of 12,400. The complex consists of a highly unstable, solvent extractable chromophore and a water soluble peptide. The apoprotein is a single chain polypeptide of 114 residues.

In the course of screening microbial organisms for new antitumor antibiotics, a novel actinomycete strain L585-6 (ATCC 53650) was selected for further investigation. This research led to the discovery of a new chromoprotein that has been named kedarcidin. The fermentation, production and biological properties of kedarcidin and the taxonomy of the producing organism were reported earlier[1]. The present paper describes the isolation, purification and physico-chemical properties of the complex.

Isolation and Purification

Extraction

The filtrate from harvested broth (10 liters) was pumped at a flow rate of 30 ml/minute through a Zeta Prep 250 QAE anion exchange cartridge, which had been washed and equilibrated with 2 liters of 50 mM Tris-HCl buffer (pH 7.4). The charged cartridge was washed with 1 liter of buffer and then eluted with 500 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 M sodium chloride. The eluate was concentrated from 500 ml to 100 ml using an Amicon ultrafiltration cell fitted with an Amicon YM5 membrane.

Purification of Kedarcidin

The above concentrate was applied to a Sephadex G-50 column (5.0×100 cm), which had been equilibrated and washed with 50 mM Tris-HCl buffer (pH 7.4). The column was developed with 2 liters of the same buffer at a flow rate of 60 ml/hour. After a forerun of 450 ml, 10 ml fractions were collected and assayed for antimicrobial activity against *Bacillus subtilis* (pH 8). The bioactive fractions ($83 \sim 133$) were pooled and concentrated to 100 ml by ultrafiltration as above. This concentrate was applied to a Trisacryl DEAE column (2.5×15 cm), which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.4). After washing with buffer (700 ml), the column was developed (300 ml) at a flow rate of 60 ml/hour with a linear gradient of sodium chloride (0.0 to 0.5 M) and buffer. Again, each fraction (5 ml) was assayed

Fig. 1. UV spectrum of kedarcidin.

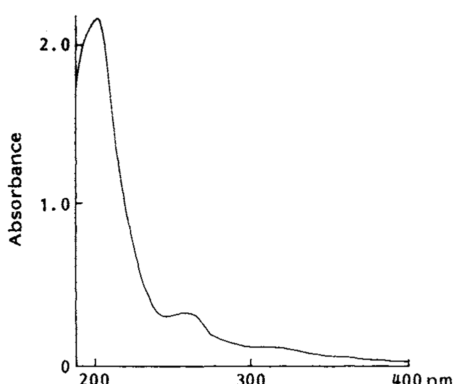


Table 1. Physico-chemical properties of kedarcidin.

Appearance	Buff colored powder
MW	12,400[a]
Isoelectric point	3.65
HPLC[b]	8.3 minutes
UV λ_{max} nm (log ε)[c]	204 (6.09), 258 (5.26), 306 (4.83), 313 (4.83)

[a] SDS polyacrylamide gel electrophoresis.
[b] Column; Waters Protein I-125, flow rate; 1.0 ml/minute, eluant; 0.2 M Tris-acetate buffer (pH 7.0), monitor; 220 nm.
[c] In water.

against *B. subtilis* (pH 8) for activity. The bioactive fractions (25~37) were pooled, desalted on a Biogel P-30 column and lyophilized to yield 200 mg of buff colored kedarcidin.

Physico-chemical Properties

Kedarcidin was isolated as a homogeneous chromoprotein complex as described above. The lyophilized solids were chemically and biologically quite stable at or below room temperature. The solids were readily soluble in water and partially insoluble in organic solvents. Neutral aqueous solutions were quite stable at 5°C but gradually decomposed at room temperature.

Kedarcidin was found to be quite an acidic material as evidenced by its isoelectric point (pI 3.65). It exhibited UV absorption maxima at 204, 258, 306 and 313 nm as shown in Fig. 1. Table 1 summarizes some of its physico-chemical properties.

HPLC, Electrophoresis and Isoelectric Focusing Assays

HPLC analyses were performed on a system consisting of a Waters Associates model 590 programmable pump, a U6K manual injector, a Waters Protein Analysis I-125 column and a Hewlett-Packard 1040A photodiode array detector system. The eluant of 0.2 M Tris-acetate buffer (pH 7.0) was pumped at a rate of 1.0 ml/minute and monitored at 220 nm. Kedarcidin gave a single peak at 8.3 minutes.

Electrophoretic analyses were performed on precast gradient (17 to 27%) polyacrylamide gels purchased from Integrated Separations Systems, Newton, MA., U.S.A. A molecular weight reference mixture consisting of peptides from cyanogen bromide treatment of myoglobin (MW 2,560, 6,380, 8,240, 14,600), myoglobin (MW 17,200), cytochrome c (MW 12,400) and lactoglobulin (MW 18,400) was used. Kedarcidin and the molecular weight markers were mixed with an equal volume of Separasol (a commercial, ready-to-use protein solubilization liquid containing sucrose and a tracking dye), heated for 3 minutes at 90°C immediately before use and applied to the gel. Analyses were run at 300V in Seprabuff (Tris-glycine-SDS, pH 8.3) until the dye reached the bottom of the gel. Protein was then visualized by staining with Coomassie Blue R-250. Kedarcidin gave a single band with an Rf the same that of cytochrome c suggesting a molecular weight of 12,400.

Isoelectric focusing gels were cast by polymerizing a solution of 29.1% acrylamide in water (10 ml), 0.9% *N,N'*-methylene-bis-acrylamide in water (10 ml), glycerol (7 ml), 1802 Ampholine pH 2.5~4 (LKB-Produkter) (3 ml) and water (quantity sufficient to equal 60 ml) with 1% ammonium persulfate

(1.5 ml) and *N,N,N',N'*-tetramethylenediamine. The electrode solutions used were 1 M phosphoric acid at the anode and a 2% 1809 Ampholine pH 6~8 at the cathode. The focusing was performed at 25 watt constant power for 2 hours. The isoelectric point of kedarcidin was thus determined to be 3.65.

Amino Acid Analysis

The amino acid composition of kedarcidin was determined by The Protein and Nucleic Acid Chemistry Facility at Yale University School of Medicine, New Haven, Conn. The procedure used was the Waters Associates Pico tag amino acid analyzer method. The results are presented in Table 2.

Amino Acid Sequence

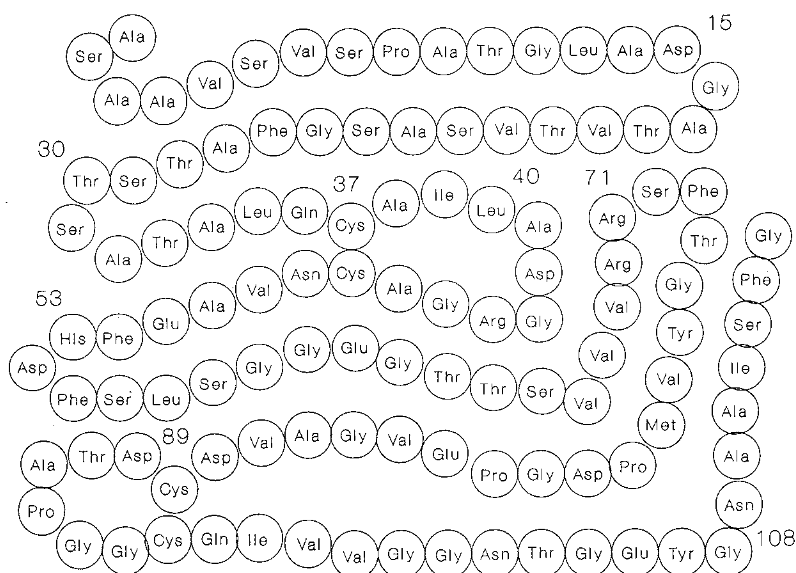
The proposed amino acid sequence of kedarcidin was determined by automated Edman degradation

Table 2. Amino acid composition.

Amino acid	Mol %	Calcd residues[a]	Found[b]	Amino acid	Mol %	Calcd residues[a]	Found[b]
Aspartic acid	8.1[c]	9.3[c]	6	Methionine	0.8	1.0	1
Asparagine	—	—	3	Isoleucine	2.5	2.9	3
Threonine	9.3	10.6	11	Leucine	3.8	4.3	4
Serine	9.3[c]	10.6[c]	12	Tryptophan	1.8	2.1	2
Glutamic acid	5.7	6.5	4	Phenylalanine	4.6	5.2	5
Glutamine	—	—	2	Histidine	1.8	2.1	1
Proline	4.7	5.9	4	Lysine	0.9	1.1	0
Glycine	16.2	18.5	18	Arginine	2.9	3.3	3
Alanine	16.3	18.6	18	Cystine	N.D.	0.0	4
Valine	11	12.5	13				

[a] Number of residues were calculated by assuming 114 residues.
[b] Number of residues determined by sequence analysis.
[c] Not corrected for hydrolytic losses.
N.D.: Not determined.

Fig. 2. Amino acid sequence of kedarcidin.



of unmodified kedarcidin and of enzymatically derived peptide fragments of *S*-pyridylethylated kedarcidin. Unambiguous identification of phenylthiohydantoin derivatives of amino acids was possible up to residue 29 of unmodified kedarcidin. Edman degradation of selected fragments derived from *S*-pyridylethylated kedarcidin obtained by digestion with trypsin, *Staphylococcus aureus* V8 protease and Asp-N protease accounted for all the residues in the protein. Appropriate overlaps of these sequences allowed for the determination of the complete sequence as shown in Fig. 2. The details of this sequence determination will be reported elsewhere[2].

Chromophore

The UV absorption maxima at 258 and 313 nm suggested the presence of a non-protein chromophore in kedarcidin. To test this hypothesis, lyophilyzed solids were triturated with 5% acetic acid in methanol. The UV spectra of the extract displayed only these two maxima. Unfortunately, this extract underwent rapid decomposition under every condition tried to remove the solvent. The insoluble apoprotein was recovered unchanged as evidenced by electrophoretic analysis and *N*-terminal amino acid sequencing.

Discussion

Bioassay guided fractionation of culture broth of a novel actinomycete strain L585-6 has led to the purification of a new chromoprotein. This compound, named kedarcidin, was found to be a water soluble acidic complex consisting of a 114 amino acid polypeptide and an extractable, highly unstable chromophore. Based upon its physico-chemical and biological properties, kedarcidin is a new member of the polypeptide, antitumor antibiotic family[3] and is most closely related to the very potent, very active antitumor antibiotics neocarzinostatin[4], auromomycin[5], macromomycin[6], actinoxanthin[7], C-1027[8] and maduropeptin[9]. Attempts to purify and chemically and biologically characterize the non-protein chromophore are in progress. The results of these studies will be reported later.

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