ISOLATION AND CHARACTERIZATION OF ANCOVENIN, A NEW INHIBITOR OF ANGIOTENSIN I CONVERTING ENZYME, PRODUCED BY ACTINOMYCETES

YASUJI KIDO, TOSHINARI HAMAKADO, TSUTOMU YOSHIDA, MASAMI ANNO and YOSHINOBU MOTOKI

Ube Research Laboratory, Fujirebio Inc., Ube, Yamaguchi 759-02, Japan

TATEAKI WAKAMIYA and TETSUO SHIBA

Department of Chemistry, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan

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Ancovenin, an inhibitor of angiotensin I converting enzyme isolated from the culture broth of a *Streptomyces* species, is a dialysable peptide composed of sixteen amino acid residues containing unusual amino acids such as *threo*-β-methyllanthionine, *meso*-lanthionine, and dehydroalanine.

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) catalyzes the conversion of angiotensin I to angiotensin II and histidylleucine. Several inhibitors of ACE have been isolated from the venom of *Bothrops jararaca*[1] and a digestion product of casein[2], *etc.* Recently, many ACE inhibitors were synthesized and their antihypertensive activities have been clinically demonstrated[3]. However, no oligo peptidyl inhibitor produced by microorganism has been reported so far.

In the course of screening of enzyme inhibitors from the microbial origin, a compound manifesting a specific inhibitory action against ACE was found in the culture broth of *Streptomyces* sp. No. A647P-2 from a soil sample collected in Hachioji City, Tokyo. The present paper describes screening, isolation, and characterization of this compound initially designated as IS83 and now named ancovenin*.

Materials and Methods

General

The IR spectrum was obtained in KBr disc using a Hitachi IR spectrophotometer Model 260-10. ^{1}H NMR spectra were obtained in D_2O with a Jeol FX-100 spectrometer. UV absorption spectra were taken with a Hitachi UV spectrophotometer Model 124. Amino acid analyses were carried out with a Hitachi KLA-5 analyzer. Fast atom bombardment mass spectrum (FAB-MS) was obtained with the Matsuda type mass spectrometer of Osaka University. The melting point is uncorrected.

Assay for ACE Inhibitory Activity

Inhibitory activities were assayed as follows. The mixture of 225 μl of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl with or without inhibitor and 200 μl of 16 mM *p*-nitrobenzoylglycylglycylglycine[4] in 0.3 M Tris-HCl buffer (pH 8.0) was preincubated in a test tube at 37°C for 3 minutes. To the mixture was added 100 μl of ACE solution and then the incubation was continued for 25 minutes. The reaction was stopped by the addition of 200 μl of 1 N hydrochloric acid. *p*-Nitro-

* A nomenclature of ancovenin is derived from the words "**an**giotensin **co**nverting **e**nzyme **in**hibitor".

benzoylglycine formed by enzymatic cleavage was extracted with 4 ml of ether. The extract was dried over Na_2SO_4 and the absorbance was measured at 268 nm. The inhibition percentage of the test sample for ACE activity was calculated from the following equation:

$$\text{Inhibition percentage} = (C-T)/(C-B) \times 100$$

where C, B and T are absorbance of enzyme control, enzyme blank and sample, respectively. One inhibition unit (IU) was defined as the dose by which the activity of ACE was inhibited in 50% [(C−B) was 1.0]. ACE used for this work was prepared from rat lungs.

Analysis of Ancovenin Using HPLC

The purity of ancovenin was monitored by HPLC and by determining the inhibitory activity against ACE. The chromatographic conditions of HPLC were as follows; column: μBondapak C_{18} (3.9 mm ID×30 cm), mobile phase: acetonitrile - water - 1 N hydrochloric acid (300: 700: 2), flow rate: 1.0 ml/minute, detection: UV absorbance detector at 210 nm. The retention time of ancovenin was 10 minutes.

Production of Ancovenin

The ancovenin-producing organism was grown at 28°C on an agar medium composed of 0.5% glucose, 0.5% Polypeptone, 0.5% meat extract, 0.25% yeast extract, 0.3% NaCl, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 1.5% agar (pH 7.2). The producing organism on the agar medium was inoculated in a 500-ml Erlenmeyer flask containing 50 ml of a medium composed of 1% glucose, 1% glycerol, 1% soybean meal, 0.25% yeast extract, 0.1% meat extract, 0.5% $(NH_4)_2SO_4$, 0.4% NaCl, 0.4% $CaCO_3$, 0.05% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, the pH being adjusted to pH 7.2 before sterilization. The seed culture was then incubated at 28°C for 4 days on a rotary shaker, and the culture (600 ml) was transferred to a 50-liter jar-fermentor containing 30 liters of a fermentation medium composed of 3% glycerol, 1.5% casein, 0.4% soybean meal, 0.25% yeast extract, 0.1% meat extract, 0.5% $(NH_4)_2SO_4$, 0.4% $CaCO_3$, 0.4% NaCl, 0.05% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.2). The cultivation was carried out at 28°C for 60 hours under agitation at 300 rpm and aeration of 30 liters/minute.

Results and Discussion

Screening and Cultivation of Ancovenin-producing Organism

After about 5,200 samples of culture broth had been assayed, *Streptomyces* sp. No. A647P-2 was found to produce an ACE inhibitor, ancovenin. The cultivation was carried out in the 50-liter jar-fermentor as described. ACE inhibitory activity of the culture filtrate reached a maximum after 60 hours. The time course of ancovenin production is shown in Fig. 1.

Purification and Physico-chemical Properties of Ancovenin

Crude ancovenin was isolated from the fermentation broth according to the procedure described in Fig. 2.

The crude ancovenin was further purified by CM-Sephadex C-25 (bufferized with 0.1 M acetate buffer at pH 4.8) column chromatography. Final purification was carried out on Bio-gel P-2 column using water as the eluent. The fractions containing the ancovenin activity were combined and lyophilized to give white solid (228 mg), $[\alpha]_D^{22}$ −45.2° (*c* 0.5, H_2O), mp 240~260°C (dec.). The product was soluble in both water and methanol, and insoluble in either acetone or ether. It gave positive reactions with ninhydrin and xanthydrol whereas negative to MOLISCH reaction. The UV absorption spectrum in water exhibited characteristic peaks for tryptophan at 250 to 300 nm. The IR spectrum was shown in Fig. 3. On a thin-layer chromatography, the substance gave Rf values as shown in Table 1.

Fig. 1. The time course of ancovenin production.

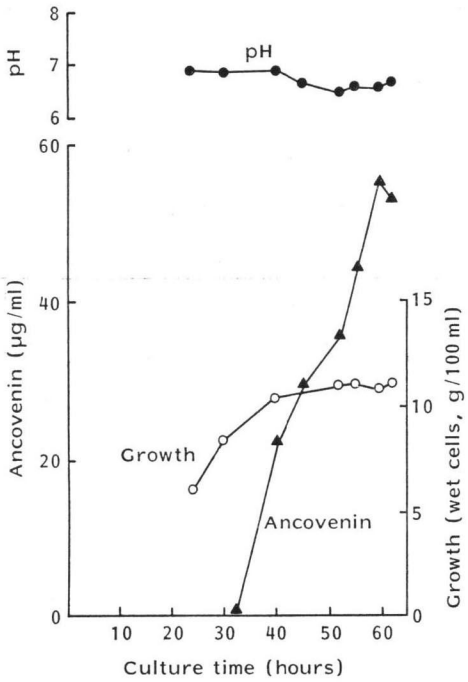
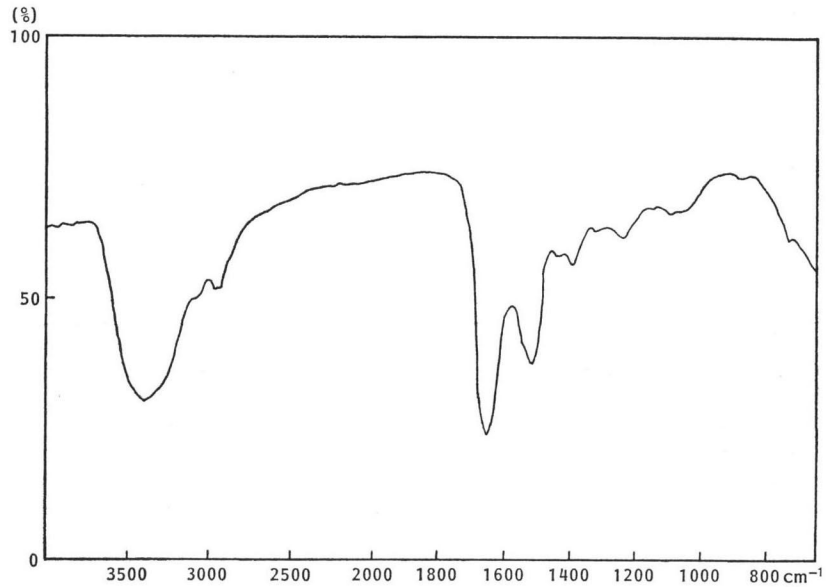


Fig. 2. Isolation and purification of ancovenin.

Culture broth (30 liters)
↓ centrifuged
Supernatant (24 liters, 138×10^6 IU)
↓ applied to a column of Diaion HP-30 (2.4 liters)
washed with water
eluted with methanol containing 0.01 N hydrochloric acid
Active fractions (2 liters, 105×10^6 IU)
↓ diluted with water (2 liters)
applied to a column of CM-Sephadex C-25 (120 ml) bufferized with acetate buffer (pH 2.5)
eluted with 0.3 M NaCl solution
Active fractions (1.5 liters, 74.8×10^6 IU)
↓ applied to a column of Diaion HP-30 (1.2 liters)
washed with water
eluted with methanol
Active fractions
↓ concentrated *in vacuo*
charged to a column of CM-Sephadex C-25 (3.1×25 cm) equilibrated with 0.1 M acetate buffer (pH 4.8)
eluted with water
lyophilized
Active material (a brown powder, 2.7 g, 45.4×10^6 IU)
↓ purified by Amberlite XAD-II column chromatography (100 mesh, 2.6×17 cm)
eluted with 20% acetonitrile
concentrated *in vacuo*
Crude ancovenin (253 mg)

Fig. 3. IR spectrum of ancovenin.



Moreover, ancovenin was dialysable and stable at pH range from 1 to 7, but labile in an alkaline solution. The molecular weight of ancovenin was determined to be 1,962 from FAB mass spectrometry.

Amino Acid Composition of Ancovenin

The amino acid composition of ancovenin was determined quantitatively by use of an automatic amino acid analyzer (Table 2). The content of tryptophan was determined by the amino acid analysis in the product hydrolyzed with constant boiling 6 N hydrochloric acid containing 4% thioglycolic acid in a sealed tube after sufficient deaeration. As shown in Table 2, in addition to ten common amino acids, two uncommon amino acids were detected. These amino acids were isolated by Dowex 50WX8 and SP-Sephadex column chromatography. One of them was crystallized from water as a free amino acid. It was found to be identical with *meso*-lanthionine in NMR spectrum as well as amino acid analysis. Another compound was identified as *threo*-β-methyllanthionine by comparison of NMR spectrum and the retention time on amino acid analyzer with an authentic sample, which was obtained from *threo*-3-methylcysteine[5,6] and β-chloroalanine in a similar manner to that in a preparation of lanthionine or *meso*-lanthionine.[7]

Table 1. Rf values of ancovenin on TLC.[a]

Solvent system	Rf value
1-Butanol - methanol - water (4: 1: 2)	0.73[b]
1-Butanol - methanol - water (4: 1: 2)	0.31[c]
1-Butanol - pyridine - acetic acid - water (15: 10: 3: 12)	0.70[c]

a) Spots were detected by spraying with ninhydrin or xanthydrol reagent and heating at 100°C.
b) Cellulose plate (Merck).
c) Silica gel 60 F_{254} (Merck).

Table 2. The amino acid composition of ancovenin.

Amino acid	Molar ratio		
	Acid hydrolysate[a]	Oxidized hydrolysate[b]	Estimated number of residues
Lys	0.97	0.86	1
Trp	0.91	—	1
Asp	1.86	2.00	2
Ser	0.90	0.98	1
Glu	0.99	0.94	1
threo-β-MeLan[c]	2.63[d]	—	2
Pro	—	1.12	1
meso-Lan[e]	0.90[f]	—	1
Gly	2.00	2.08	2
Val	1.07	0.81	1
Leu	1.07	1.07	1
Phe	0.98	0.96	1
Dha[g]	—	—	1

a) The sample was hydrolyzed for 24 hours at 110°C in a sealed tube.
b) The acid hydrolysate of ancovenin was oxidized with performic acid. *threo*-β-Methyllanthionine and lanthionine derived from *meso*-lanthionine having the same retention time as that of proline were changed to sulfoxide or sulfone derivatives which were eluted faster than aspartic acid.
c) *threo*-β-Methyllanthionine.
d) The molar ratio of this amino acid was calculated by the subtraction of overlapped amino acids, *i.e.*, 1.00 mole of proline determined from the analysis of oxidized hydrolysate[b] and 0.22 mole of lanthionine derived from *meso*-lanthionine as a result of epimerization[f].
e) *meso*-Lanthionine.
f) The value was corrected taking into account of a epimerization of *meso*-lanthionine during the acid hydrolysis. The hydrolysis of pure *meso*-lanthionine for 24 hours caused epimerization of 24%.
g) Dehydroalanine. The presence of this amino acid was clearly ascertained from a new formation of one molar alanine in the hydrolysis of ancovenin after catalytic hydrogenation as well as a calculation of the molecular weight of ancovenin.

Although ancovenin contains unique sulfide amino acids, *i.e.*, *meso*-lanthionine and *threo*-β-methyllanthionine which are found in peptide antibiotics such as nisin[8,9], subtilin[10,11], duramycin[12], gardimycin[13], and cinnamycin[14], it did not show any antibacterial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* IFO 12732 and so on even in a dose of 1,000 μg/ml.

Details about the antibacterial and ACE inhibitory activities* will be reported in a following paper. The structure of ancovenin is currently under investigation.

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* In the preliminary tests, the IC_{50} value of ancovenin to rat lung ACE is 8.5×10^{-8} M, while the values of captopril, MK-422, and potentiator C, a peptidyl inhibitor, are 1.4×10^{-8} M, 3.5×10^{-9} M, and 5.6×10^{-7} M, respectively.