K-13, A NOVEL INHIBITOR OF ANGIOTENSIN I CONVERTING ENZYME PRODUCED BY *MICROMONOSPORA HALOPHYTICA* SUBSP. *EXILISIA*

I. FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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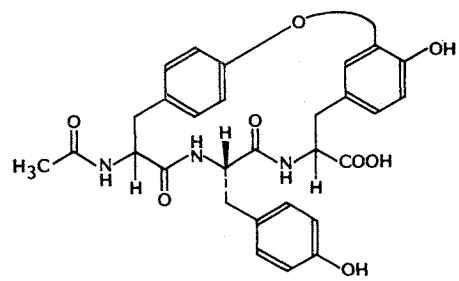
A novel inhibitor of angiotensin I converting enzyme (ACE), designated K-13, was isolated from the culture broth of *Micromonospora halophytica* subsp. *exilisia* K-13. K-13 inhibited ACE non-competitively when hippuryl-L-histidyl-L-leucine was used as a substrate. The inhibition constant (*Ki*) was 0.349 μM. K-13 hardly inhibited carboxypeptidase A, trypsin, α-chymotrypsin, leucine aminopeptidase, and aminopeptidase B even at a level of 61 μM. When K-13 was administered intravenously to rats, it inhibited the pressor response to angiotensin I.

In previous papers, we reported novel and potent inhibitors of angiotensin I converting enzyme (ACE) from microbial origin, K-26[1] and K-4[2]. During further screening for ACE inhibitors, we found that an actinomycete strain identified as *Micromonospora halophytica* subsp. *exilisia* K-13 produced a new ACE inhibitor, K-13 (Fig. 1).

In this communication, we report the fermentation, isolation, physico-chemical and biological properties of K-13. The taxonomic studies of the producing organism and structure elucidation of K-13[3] will be published in separate papers.

Fig. 1. The structure of K-13.



Materials and Methods

Materials

Carboxypeptidase A (bovine pancreas) was obtained from Sigma; carboxypeptidase B (porcine pancreas) and leucine aminopeptidase (hog kidney) from Boehringer Mannheim, and α-chymotrypsin (bovine pancreas) and trypsin (bovine pancreas) from Worthington Biochem. Hippuryl-L-histidyl-L-leucine (HHL) and angiotensin I were purchased from Protein Research Foundation, Osaka; hippuryl-L-phenylalanine, L-arginyl β-naphtylamide, and L-leucine β-naphthylamide from Sigma, and benzoyl-L-tyrosine ethyl ester and *p*-tosyl-L-arginine methyl ester hydrochloride were from Nakarai Chemicals, Ltd.

Microorganisms

Micromonospora halophytica subsp. *exilisia* K-13, isolated from a soil sample was used in this experiment.

Medium and Culture

The seed medium consisted of glucose 1.0%, soluble starch 1.0%, beef extract 0.3%, yeast extract 0.5%, Bacto-tryptone (Difco) 0.5% and $CaCO_3$ 0.2%, pH 7.2. The production medium consisted

of dextrin 3.0%, soybean meal 2.0%, corn steep liquor 0.25%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03% and $CaCO_3$ 0.3%, pH 7.8.

Four milliliters of seed medium in a test tube was inoculated with a loopful of microorganisms grown on the surface of an agar slant. The test tube was incubated for 4 days at 28°C on a reciprocal shaker. One milliliter of this culture was transferred into a 50-ml test tube containing 10 ml of the seed medium. The test tube was then incubated for 1 day at 28°C on a reciprocal shaker. The seed culture (50 ml) was transferred into 500 ml of the seed medium in 2-liter flask. After incubation for 1 day at 28°C on a rotary shaker (220 rpm), 1.5 liters (3 flask content) of the seed culture were transferred into a 30-liter jar fermentor containing 18 liters of the production medium. The fermentor was operated for 68 hours at 30°C with agitation at 350 rpm and aeration of 18 liters/minute.

Measurement of Enzyme Activities

ACE activity was measured as described previously[2]. Briefly, the assay mixture (total volume, 300 μl) consisting of 3 mM HHL, 0.3 M NaCl, 50 mM sodium borate buffer, pH 8.3, and 50 μl of ACE prepared from rabbit lung[2] (*ca.* 10 units) with or without inhibitors was incubated for 30 minutes at 37°C. The reaction was terminated by the addition of 250 μl of 1 N HCl and the hippuric acid formed was extracted by 1.5 ml ethyl acetate. The ethyl acetate layer (1 ml) was evaporated at 120°C for 30 minutes and the residue was dissolved in 3.0 ml of 50 mM sodium borate buffer, pH 8.3. The hippuric acid was quantified from its absorbance at 228 nm.

The assay of carboxypeptidase A, trypsin, and α-chymotrypsin was described in a previous paper[2]. Leucine aminopeptidase was assayed by the method of HOPSU *et al.*[4] by use of L-leucine β-naphthylamine as a substrate. Aminopeptidase B was measured according to the method of HOPSU *et al.*[4] by use of L-arginine β-naphthylamine as a substrate and the enzyme prepared from rat liver by the method of HOPSU *et al.*[4].

One unit of ACE activity was defined as the amount of enzyme which catalyzed 1 μmol of substrate/minute at 37°C.

Measurement of the Pressor Response to Angiotensin I in Rats

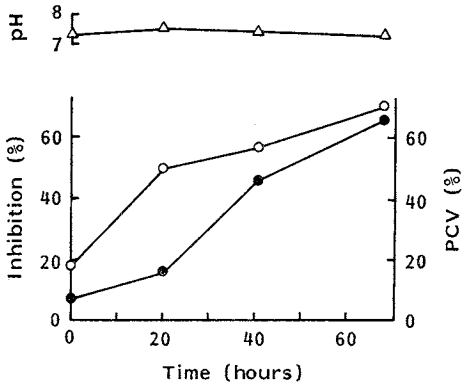
Male Sprague-Dawley rats, weighing about 350 g, were anesthetized with urethane (1.5 g/kg, ip). Blood pressure was measured with a pressure transducer (Nihon Kohden, MPU-0.5A) through polyethylene cannula placed in the carotid artery and recorded on a polygraph (Nihon Kohden RM-600). Angiotensin I was injected into the jugular vein. K-13 was injected from the tail vein.

Results

Fermentation

A time course of the fermentation of K-13 is shown in Fig. 2. Total inhibitory activities towards ACE increased rapidly during early logarithmic phase of growth and then reached a maximum level at 60~68 hours.

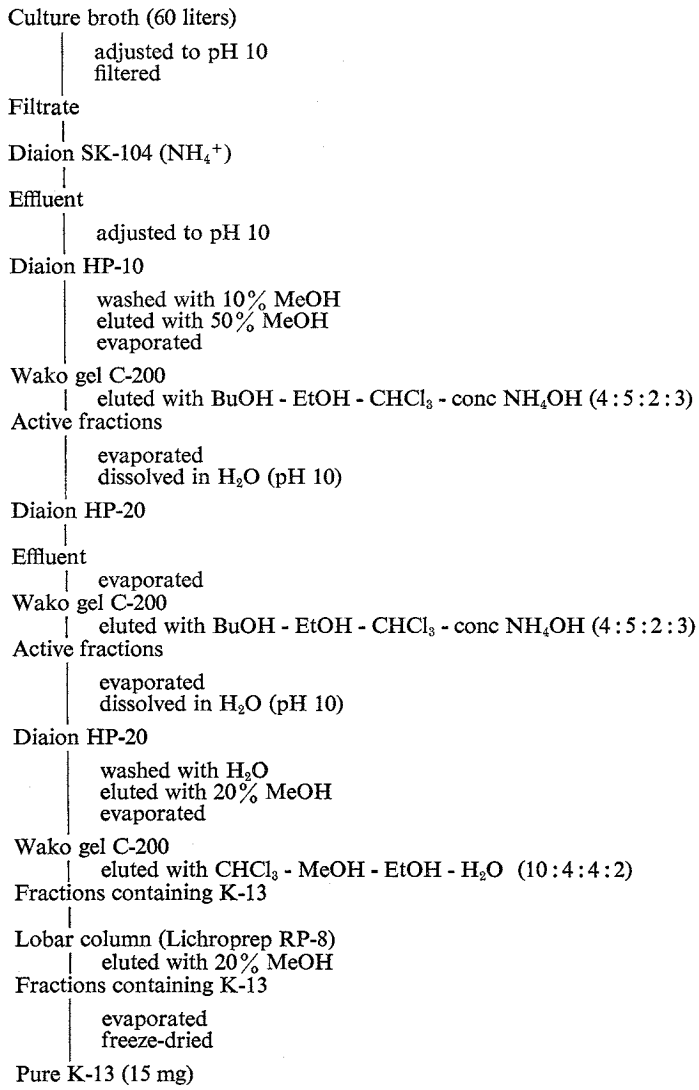
Fig. 2. Time course of K-13 fermentation.
Micromonospora halophytica subsp. *exilisia* K-13.
The ordinate indicate the inhibition percent of ACE activity by 10 μl of the culture broth.
● PCV: Packed cell volume, ○ inhibition, △ pH.



Isolation and Purification

K-13 was isolated from the culture broth of *Micromonospora halophytica* subsp. *exilisia* K-13 by repeated column chromatography with Diaion SK-104, Diaion HP-10, Wako gel C-200 and Lichroprep RP-8. The flow diagram for the isolation is shown in Fig. 3. The yield of pure K-13 was 15 mg from 60 liters of culture broth.

Fig. 3. Isolation of K-13.



Physico-chemical Properties of K-13

K-13 was obtained as white powder, melting at 265~270°C with decomposition. The compound is water soluble and positive to Rydon-Smith and negative to ninhydrin. The Rf values on silica gel TLC developed with the upper layer of BuOH - PrOH - H_2O (2 : 1 : 3), BuOH - EtOH - $CHCl_3$ - conc NH_4OH (4 : 5 : 2 : 5), $CHCl_3$ - MeOH - EtOH - H_2O (10 : 4 : 4 : 2), and $CHCl_3$ - MeOH (9 : 1) were 0.62, 0.53, 0.40 and 0.00, respectively.

Biological Properties of K-13

K-13 potently inhibited ACE in a dose dependent manner. The concentration of K-13 causing 50% inhibition of ACE was 177 ng/ml. As shown in Fig. 4, K-13 non-competitively inhibited ACE. The inhibition constant (*Ki*) was 0.349 μM.

Effect of K-13 on carboxypeptidase A, α-chymotrypsin, trypsin, leucine aminopeptidase, and

Fig. 4. Inhibition of angiotensin I converting enzyme by K-13.
(a) Lineweaver-Burk plots, (b) Dixon plots.
K-13: ▲ 0.548, △ 0.274, ● 0.137, ○ 0 μM.

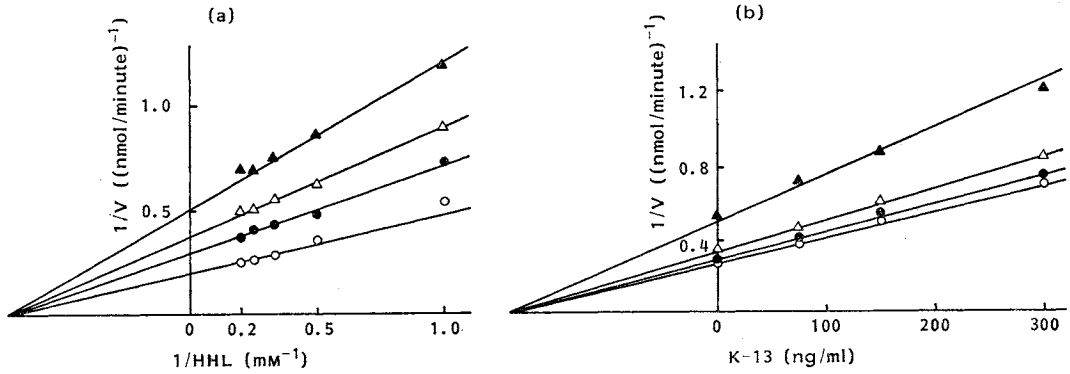


Fig. 5. Effect of K-13 on the pressor response to angiotensin I in an anesthetized, normotensive rat.
Each drug was administered at the time indicated by an arrow at the following dose: angiotensin I (AG I) 0.3 μg/kg; K-13, 3 mg/kg.

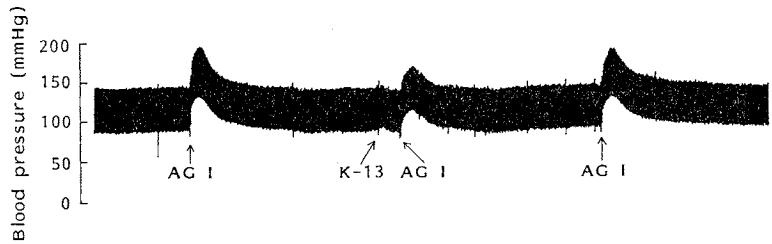


Table 1. Effect of K-13 on various enzymes.

Enzyme	Concentration of K-13 (μM)	Inhibition (%)
Carboxypeptidase A	122	0
Trypsin	122	0
α-Chymotrypsin	122	0
Leucine aminopeptidase	73	6.6
Aminopeptidase B	61	5.3

aminopeptidase B was examined and the results are summarized in the table. High concentrations (61 ~ 122 μM) of K-13 hardly inhibited these enzymes, indicating that K-13 was a specific inhibitor of ACE.

Fig. 5 shows the effect of K-13 on the pressor response to angiotensin I in rats. K-13 previously administered intravenously (3 mg/kg) inhibited the pressor response to angiotensin I (59%).

Discussion

Many microbial inhibitors of ACE including aspergillosamines[5], muraceins[6], L-681,176[7], I5B2[8], ancovenin[9], A58365A[10], phenacein[11], WF-10129[12] and foroxymithine[13] have recently been reported. In our screening of ACE inhibitors of microbial origin, we found novel and potent compounds, K-26 and K-4, and described them in previous papers[1,2]. Here, we report a novel ACE inhibitor K-13 produced by *Micromonospora halophytica* subsp. *exilisia* K-13. K-13 specifically inhibited ACE with a *Ki* value of 3.49×10^{-7} M. The structure of K-13 (Fig. 1) resembles that of OF4949[11], a potent inhibitor of aminopeptidase B produced by *Penicillium rugulosum* OF4949. However, K-13 (61 μM) hardly inhibited aminopeptidase B (Table 1).

K-4 and K-26 inhibited ACE non-competitively when HHL was used as a substrate[1,2]. Similarly, K-13 was shown to be a non-competitive inhibitor. The hypothetical model of the binding site on ACE of captopril, a competitive inhibitor of ACE, was proposed by CUSHMAN *et al.*[14,15]. It may be interesting to explore the binding sites on ACE of these non-competitive inhibitors, and compare them with those of captopril.

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