K-26, A NOVEL INHIBITOR OF ANGIOTENSIN I CONVERTING ENZYME PRODUCED BY AN ACTINOMYCETE K-26

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A novel inhibitor of angiotensin I converting enzyme (ACE), designated K-26, was isolated from the broth filtrate of an actiomycete K-26. K-26 is a water soluble, acidic peptide composed of an equal mol of L-isoleucine, L-tyrosine and 1(*R*)-1-amino-2-(4-hydroxyphenyl)-ethylphosphonic acid. The IC_{50} of K-26 for ACE inhibition was 6.7 ng/ml when hippuryl-L-histidyl-L-leucine was used as a substrate of ACE. K-26 possesses hypotensive activity *in vivo*.

Angiotensin I converting enzyme (ACE) has been found to play a critical role in the regulation of blood pressure in man and animals. Ever since the discovery of captopril[1], a specific inhibitor of ACE, and its successful application to the therapy of hypertension, attempts have been made to develop ACE inhibitors as antihypertensive agents of clinical use and a growing number of inhibitors of ACE, synthetic or microbial, have been discovered in recent years. During the course of screening for microbial inhibitors of ACE in our laboratory, an active substance, designated K-26[2], was isolated from the broth filtrate of an actinomycete K-26 and characterized chemically and biologically.

In this paper, the production, isolation, physico-chemical properties and biological activity both *in vivo* and *in vitro* of K-26 are described. The structure elucidation studies will be described in a separate paper[3].

Materials and Methods

General

The IR spectrum was recorded on a Shimadzu IR spectrophotometer Model IR-27G. The UV spectrum was obtained on a Hitachi UV spectrophotometer Model 124. The amino acid analysis was carried out with a Jeol JLC-200A amino acid analyzer. The rotation was measured on a Perkin-Elmer model 141 polarimeter.

Preparation of ACE

All procedures were performed below 4°C. One gram of rabbit lung acetone powder was suspended in 10 ml of 50 mM potassium phosphate buffer, pH 8.3, and stirred gently overnight. The suspension was then centrifuged at 24,000×*g* for 40 minutes. The clear supernatant was used for the assay of ACE activity. For kinetic analysis, the crude ACE was purified 250-fold according to the method of CHEUNG *et al.*[4]. Enzyme units were expressed as μmol of hippuryl-L-histidyl-L-leucine (HHL) hydrolyzed per minute. Protein was determined by the method of LOWRY *et al.*[5].

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Assay of ACE Inhibitory Activity

ACE activity was assayed in the presence or absence of an inhibitor by the method of CUSHMAN and CHEUNG[6], HHL being used as a substrate, or by that of CHEUNG and CUSHMAN[7] when angiotensin I (AG I) was used as a substrate. IC_{50} was defined as the concentration at which ACE activity was inhibited by 50%.

Assay of Other Proteases

Pancreatic carboxypeptidase A, α-chymotrypsin and trypsin were assayed spectrophotometrically by hydrolysis of hippuryl-L-phenylalanine[8], *N*-benzoyl-L-tyrosine ethyl ester (BTEE)[9] and *p*-toluenesulfonyl-L-arginine methyl ester (TAME)[9], respectively. Carboxypeptidase B was assayed likewise by hydrolysis of hippuryl-L-arginine[10].

Culture and Medium Conditions

The producing organism, an actinomycete K-26 (FERM-P 5859, NRRL 12379), was recently isolated from a soil sample of the bank at Kadoike in Numazu City, Shizuoka Prefecture. K-26 was produced also by *Micromonospora* sp. strain K-24 (FERM-P 7031). The strain K-26 was used in this study. Morphological, cultural and physiological characteristics of the strains will be described in detail elsewhere. The seed medium consisted of glucose 1%, soluble starch 1%, beef extract 0.5%, yeast extract 0.5%, Bacto Tryptone 0.5% and $CaCO_3$ 0.2%, pH 7.2. The production medium consisted of soluble starch 4%, soy bean meal (SBM) 3%, corn steep liquor (CSL) 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03% and $CaCO_3$ 0.3%, pH 7.8.

Ten milliliters of seed medium in a 50-ml test tube was inoculated with a loopful of microorganism grown on the surface of an agar slant. The test tube was then incubated for 10 to 12 days at 28°C on a reciprocal shaker. Three milliliters of this culture was transferred into a 300-ml flask containing 30 ml of seed medium. The flask was then incubated for 3 to 4 days at 28°C on a rotary shaker (220 rpm). Five milliliters of this seed culture was transferred into a 300-ml flask containing 50 ml of production medium. The flask was then incubated for 5 days at 28°C on a rotary shaker (220 rpm). When a jar fermentor was employed in the production of K-26, 10 ml of the seed culture incubated in a 50-ml test tube for 10 to 12 days at 28°C was transferred into a 2-liter flask containing 300 ml of seed medium. The flask was incubated for 3 to 4 days at 28°C on a rotary shaker (220 rpm). The seed culture in three flasks was combined and transferred into a 30-liter jar fermentor containing 15 liters of production medium. The fermentor was operated for 5 days at 30°C with agitation at 350 rpm and aeration of 15 liters/minute. The growth was monitored by packed cell volume (PCV). The production of K-26 was traced by measuring ACE inhibitory activity of the broth filtrate as described above.

Measurement of Pressor Responses to Angiotensin I, Angiotensin II and Noradrenaline in Anesthetized Rats

Male Wister strain rats weighing 200 to 250 g were used. All rats were anesthetized by intraperitoneal administration of urethane at 500 mg/kg and α-chloralose at 50 mg/kg. Carotid artery and femoral vein were cannulated with polyethylene tubings. The arterial canula was connected to a pressure transducer (MPV-0.5, Nihon Kohden), and the blood pressure was recorded on polygraph (RM-45, Nihon Kohden). Drugs were dissolved in saline, and injected into the venous canula.

Materials

Peptides and amino acid derivatives were purchased from the following commercial sources: HHL, BTEE and TAME from Calbiochem; hippuryl-L-arginine and hippuryl-L-phenylalanine from Sigma Chemical Corp.; AG I from Protein Res. Foundation, Osaka, Japan. Rabbit lung acetone powder was obtained from Pel-Freez Biologicals; carboxypeptidase A, thermolysin, pronase E, papain type II, pepsin and subtilisin BPN' from Sigma Chemical Corp.; carboxypeptidase B and leucine aminopeptidase from Boehringer-Mannheim; trypsin and α-chymotrypsin from Worthington Biochemical Corp. Other chemicals were reagent grade and commercially available.

Results

Production of K-26 by Fermentation

A time course of K-26 production by the producing organism in a 30-liter jar fermentor is represented in Fig. 1. The amount of K-26 produced increased after the growth of cells reached the maximum. The effect of natural nitrogen sources, the concentration of phosphate and amino acids added on the production of K-26 was investigated. Corn steep liquor at the concentration of 0.5, 1 and 2% stimulated the production, but repressed it at 5% or when used in combination with 3% soy bean meal (Table 1-A, B). K-26 production was remarkably affected by phosphate concentration. The optimum concentration of phosphate for the production was 0.05% as shown in Fig. 2. The effect of amino acids is summarized in Table 2. The addition of serine at 0.5% slightly increased the amount of K-26 produced while other amino acids tested had no effect or decreased it.

Fig. 1. Time course of K-26 production.

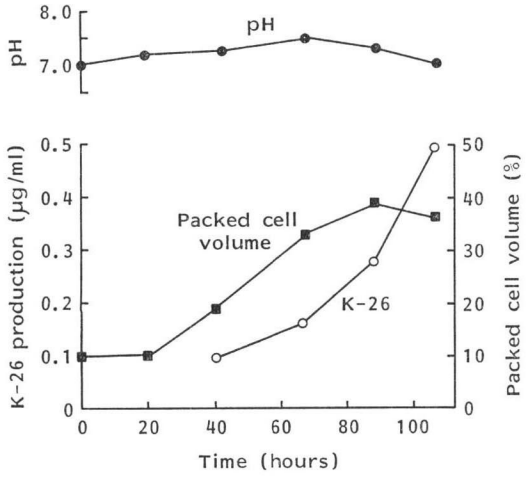


Fig. 2. Effect of the concentration of phosphate on K-26 production.

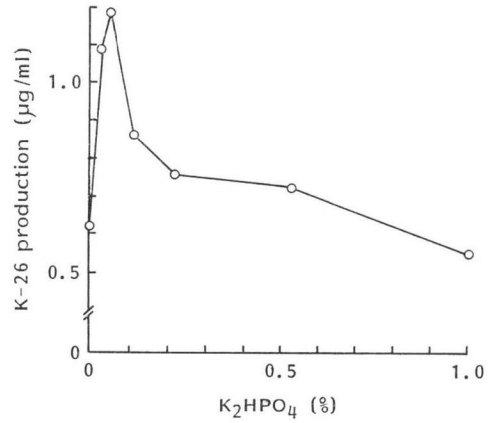


Table 1. Effect of natural nitrogen sources on the production of K-26.

A

Nitrogen-sources	K-26 produced (μg/ml)
Soy bean meal (SBM)	0.45
Corn steep liquor (CSL)	0.66
Soluble vegetable protein	0.36
Pharmamedia	0.18
Polypeptone	0.14
Meat extract	*
Yeast extract	*
Ebios	0.13
NZ-Amine	*
Casamino acids	*
Bacto-Tryptone	0.16
Control	0.35

B

Nitrogen-sources (%)		K-26 produced (μg/ml)
SBM, 3 plus CSL,	0	0.52
	1	0.45
	2	0.26
	5	0.12
CSL,	0.5	1.4
	1	0.55
	2	0.66
	5	0.13
Control		0.5

* Negligible.

SBM (3%) plus CSL (0.5%) in the control medium were replaced by 2% of various natural nitrogen sources (Table 1-A) and by SBM (3%) plus CSL (0, 1, 2, 5%) or CSL alone (0.5, 1, 2, 5%) (Table 1-B).

The incubation was carried out in the same way as described in Materials and Methods.

Table 2. Effect of amino acids on the production of K-26.

Amino acids	K-26 produced (μg/ml)
Glycine	*
Alanine	0.42
Valine	0.11
Leucine	0.55
Isoleucine	0.32
Serine	0.63
Phenylalanine	0.3
Tyrosine	0.39
Tryptophan	0.15
Methionine	0.15
Cysteine	0.1
Proline	0.33
Aspartic acid	*
Glutamic acid	*
Histidine	0.14
Lysine	0.42
Arginine	0.32
None (control)	0.54

* Negligible.

Amino acids were added at 0.5% to the production medium. The incubation was carried out in the same way as described in Materials and Methods.

Fig. 3. Isolation procedures.

Broth filtrate
↓ pH adjusted to 3.0
Diaion HP-10
↓ washed with H_2O and eluted with 50% MeOH
↓ concd *in vacuo* and pH adjusted to 7.0
Diaion HPA-10 (Cl^-)
↓ washed with H_2O and eluted with 1 M NaCl
↓ pH adjusted to 2.0
Diaion HP-10
↓ washed with H_2O and eluted with 50% MeOH
↓ concd *in vacuo* and pH adjusted to 2.0
Extracted with BuOH
↓ concd *in vacuo* and H_2O added
Diaion SK-104 (H^+)
↓ concd *in vacuo* and freeze-dried
↓ dissolved into 0.15 M potassium phosphate, buffer pH 7.5 (PBS)
DEAE-Sephadex A-25 (buffered in PBS)
↓ eluted with PBS
↓ pH adjusted to 2.0
Diaion HP-10
↓ washed with H_2O and eluted with 50% MeOH
↓ concd *in vacuo*
Sephadex LH-20
↓ eluted with 50% MeOH
↓ concd *in vacuo* and freeze-dried
Silica gel
↓ developed with the upper layer of BuOH - PrOH - H_2O, 2: 1: 3
↓ concd *in vacuo* and freeze-dried
White powder of K-26

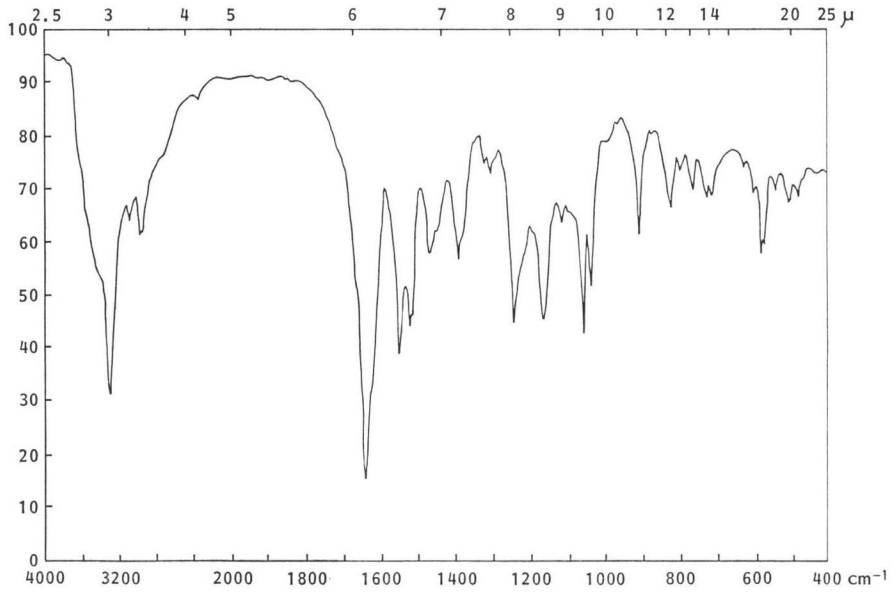
Purification and Isolation

Isolation procedures are outlined in Fig. 3. Thirty liters of borth filtrate, adjusted to pH 3.0, was passed through a 2-liter column of Diaion HP-10. The column was washed with water and eluted with 6 liters of 50% aqueous methanol solution. The eluate was concentrated *in vacuo* to 3 liters and passed through a 300-ml column of Diaion HPA-10 (Cl^-) at pH 7.0. The column was washed with water and eluted with 1 liter of 1 M NaCl solution. The eluate was then applied to a 100-ml column of Diaion HP-10 at pH 2.0 for desalting. The column was washed with water and eluted with 500 ml of 50% aqueous methanol solution. The eluate was concentrated *in vacuo* to 200 ml and extracted three times with butanol at pH 2.0. The butanol layer was evaporated to dryness and dissolved in 200 ml of water. The solution was then passed through a 100-ml column of Diaion SK-104 (H^+). The effluent fraction was collected, concentrated and freeze-dried to get brownish crude powder of K-26. The powder was dissolved into a small portion of 0.15 M potassium phosphate buffer solution, pH 7.5 (PBS) and placed on a 100-ml column of DEAE-Sephadex A-25 bufferized in PBS. The column was eluted with the same buffer. The active fractions were collected, adjusted to pH 2.0 and treated with a 50-ml column of Diaion HP-10 for desalting. The eluate of Diaion HP-10 was concentrated and freeze-dried to obtain partially purified pale yellow powder. It was further chromatographed on a column of Sephadex LH-20 (2.0×90 cm) in 50% aqueous methanol solution and finally purified on a column of silica gel

Table 3. Physico-chemical properties of K-26.

Appearance		White powder
Nature		Acidic
MP		>300°C (dec)
Optical rotation; $[\alpha]_D^{25}$		−4.8° (*c* 0.1, H_2O)
UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	Acidic & neutral:	278 (46), 281 (sh)
	Alkaline:	294 (56.5)
Color reactions	Positive:	Rydon-Smith, I_2
	Negative:	Ninhydrin, anthrone, Ehrlich, aniline-diphenylamine, nitroprusside
Solubility	Soluble in:	H_2O
	Insoluble in:	EtOH, AcOEt, $CHCl_3$, benzene
Composition of amino acid		Isoleucine, tyrosine, 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid

Fig. 4. IR spectrum of K-26 (KBr).



(Wakogel, 1.8×80 cm) developed with the upper layer of butanol - propanol - water (2: 1: 3). The active fractions were pooled, concentrated *in vacuo* and freeze-dried to get 15 mg of purified white powder of K-26.

Physico-chemical Properties

Physico-chemical properties are represented in Table 3. K-26 is a water soluble, acidic substance which is positive to Rydon-Smith and iodine but negative to ninhydrin and all other color reactions tested. The IR spectrum of K-26 suggests the presence of amide group in its structure (Fig. 4). The UV spectrum was quite identical to that of tyrosine. Acid hydrolysis of K-26 with 6 N HCl at 110°C for 16 hours gave isoleucine, tyrosine and an unknown ninhydrin-positive compound (Fig. 5). Amino acid analysis of the acid hydrolysate revealed that the molar ratio of isoleucine and tyrosine was 1: 1 and that the unknown compound was eluted between serine and glutamic acid (the data not shown here). It is clear from these results that the unknown compound is different from proline and any

Fig. 5. Thin-layer chromatogram of acid hydrolysate of K-26 and some authentic amino acids.
Adsorbent: cellulose.
Detection: ninhydrin.
Solvent: BuOH - AcOH - H_2O (4: 1: 1).
Sample: 1; acid hydrolysate of K-26, 2; Leu, 3; Ileu, 4; Val, 5; Tyr, 6; Pro, 7; Glu.

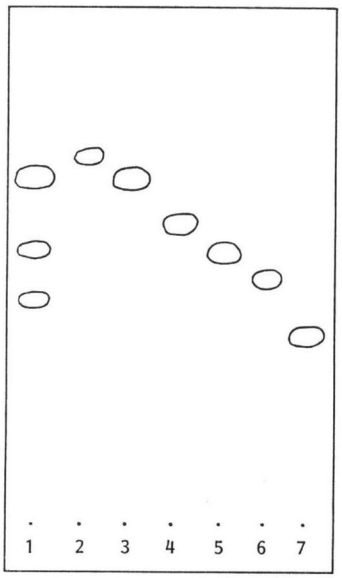


Table 4. ACE inhibitory activity *in vitro* of K-26.

Substrate	IC_{50} (ng/ml)	
	Phosphate	Borate
HHL	6.7 (6.0)	12 (12)
AG I	3.3 (5.0)	—

( ): IC_{50} of captopril.
—: Not tested.
ACE inhibitory activity was determined both in 0.1 M phosphate buffer, pH 8.3, and 0.1 M borate buffer, pH 8.3, HHL or AG I being used as a substrate.
Six determinations were made to obtain an IC_{50}. The average values were represented here.

Table 5. Inhibitory activity of K-26 against some proteases.

Proteases	Inhibition (%)
ACE	100
Carboxypeptidase A	13.7
Carboxypeptidase B	0
α-Chymotrypsin	0
Trypsin	0

ACE and proteases were assayed in the presence of 33 μg/ml of K-26 by the methods described in Materials and Methods.

other natural amino acids. The structure of the unknown compound was deduced to be 1(*R*)-1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid. Isoleucine and tyrosine were found to have L-configuration. The molar ratio of three components was determined to be 1: 1: 1. The DNP-derivative of K-26 was not obtained, suggesting that the *N*-terminal was modified. The *C*-terminal residue was determined to be 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid by the hydrazinolysis of K-26. Details of these studies will be described in a separate paper[3].

Biochemical Properties

ACE inhibitory activity *in vitro* of K-26, together with that of captopril, is shown in Table 4. When HHL was used as a substrate, IC_{50} values were 6.7 ng/ml in a phosphate buffer and 12 ng/ml in a borate buffer. When AG I was a substrate, the IC_{50} was 3.3 ng/ml. K-26 proves to be just as potent as captopril in ACE inhibition *in vitro*. Table 5 shows the inhibitory activity of K-26 against some proteases. At the concentration of 33 μg/ml which is 5,000 times higher than the IC_{50} of K-26 for ACE inhibition, this compound inhibited carboxypeptidase A only slightly but did not inhibit other proteases (carboxypeptidase B, α-chymotrypsin and trypsin) at all, implying that K-26 is a specific inhibitor of ACE. K-26 was not hydrolyzed by any of the proteases so far tested (carboxypeptidases A and B, trypsin, α-chymotrypsin, leucine aminopeptidase, pronase, pepsin, papain, thermolysin and substilisin).

Kinetics of ACE Inhibition by K-26

The kinetic study was carried out with purified ACE in 0.1 M borate buffer, pH 8.3, HHL being

Fig. 6. Lineweaver-Burk plot for ACE inhibition by K-26.
K-26 concentration: ○; 0 ng/ml, ●; 11.1 ng/ml, ▲; 22.2 ng/ml.

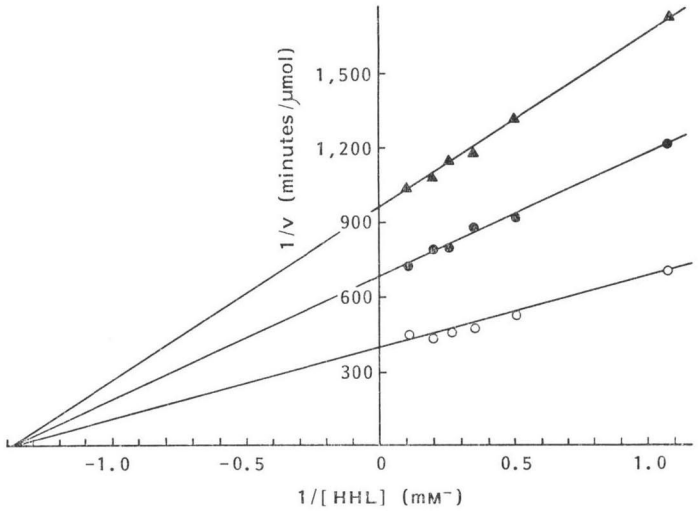
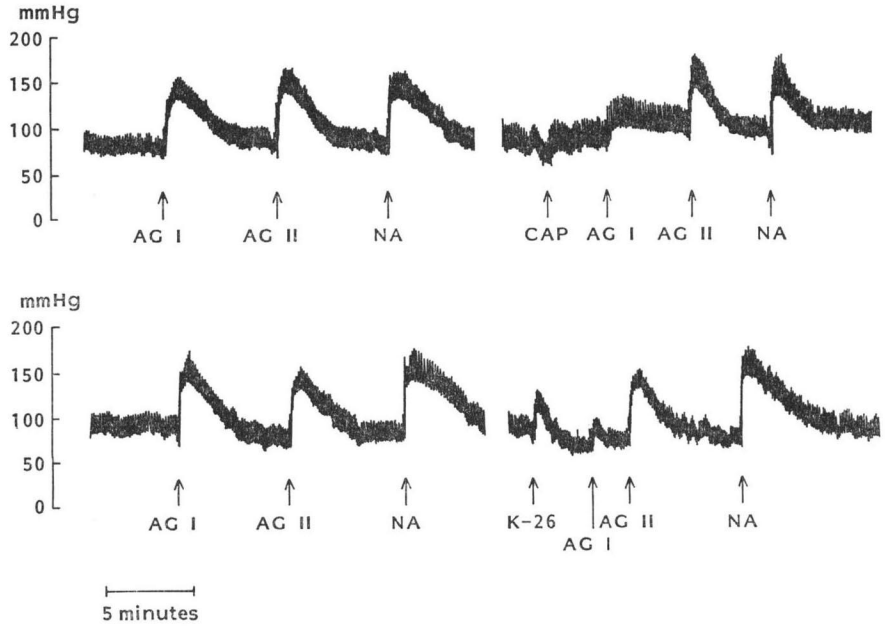


Fig. 7. Effect of K-26 or captopril on the pressor response to angiotensin I, angiotensin II and noradrenaline in an anesthetized, normotensive rat.

Each drug was administered intravenously at the time indicated by an arrow at the following dose: angiotensin I (AG I); 300 ng/kg, angiotensin II (AG II); 100 ng/kg, noradrenaline (NA); 3 μg/kg, K-26; 0.1 mg/kg, captopril (CAP); 0.1 mg/kg.

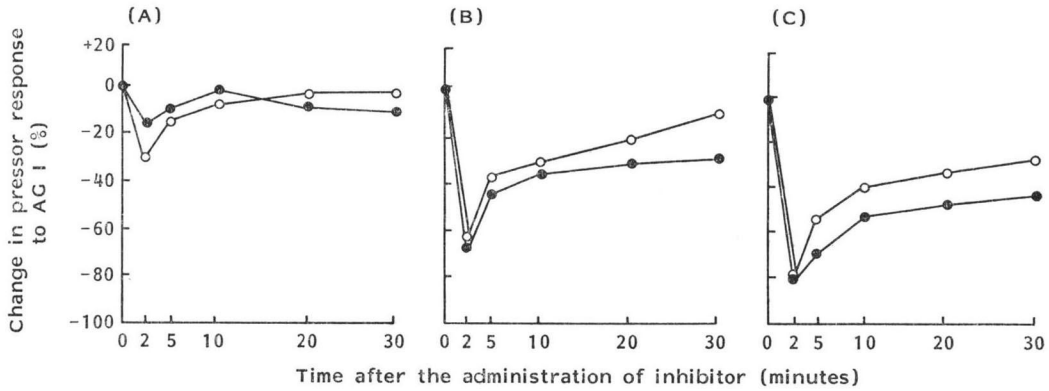


employed as a substrate. As is seen from the Lineweaver-Burk plot for ACE inhibition, K-26 inhibited ACE non-competitively with respect to HHL, *Km* and *Ki* being 0.74 mM and 0.03 μM, respectively.

Biological Activity

The biological activity *in vivo* of K-26 was determined with captopril as a control. In an an-

Fig. 8. Inhibition of pressor response to AG I by K-26 or captopril in an anesthetized, normotensive rat. Both inhibitors were administered intravenously at 0.01 (A), 0.03 (B) and 0.1 (C) mg/kg. AG I, at 300 ng/kg. ●; K-26, ○; captopril.



esthetized, normotensive rat, the pressor response to AG I was antagonized completely and specifically by K-26 administered intravenously at a dose of 0.1 mg/kg (Fig. 7). The pressor response to AG II or noradrenaline was not inhibited at all. The dose dependence and duration of the inhibition by K-26 of the pressor response to AG I was investigated also in an anesthetized, normotensive rat. K-26, administered intravenously at 0.01 to 0.1 mg/kg, showed the same degree of inhibition and duration as those of captopril (Fig. 8).

Discussion

Several microbial inhibitors of ACE have been reported recently. They include aspergillomarasmines[11], muraceins[12], L-681,176[13], I5B2[14], A58365A, B[15], ancovenin[16] and talopeptin[17]. They are peptides or peptide-like compounds except that A58365A and B are bicyclic dicarboxylic acids. Some of them have considerable ACE inhibitory activity, but others are less potent as ACE inhibitors. K-26 is a new member of microbial ACE inhibitors and possesses the highest inhibitory activity *in vitro* among them, being comparable to captopril. K-26 is unique in following respects. 1) It does not contain a proline residue which has been reported to be essential for the interaction of an inhibitor with ACE[18] and is a structural component of almost all potent inhibitors of ACE. Nevertheless, it is highly active against ACE *in vitro* as described above. 2) It contains an unusual aminophosphonic acid, 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid. The phosphonic moiety of this compound may act as the ligand for the active site zinc ion on ACE. 3) It inhibited ACE non-competitively with respect to HHL whereas captopril and most others inhibited ACE competitively. There are only a few non-competitive inhibitors of ACE so far reported, among which are K-4[19], N^{α}-phosphoryl-L-Ala-L-Pro, N^{α}-phosphoryl-L-Val-L-Trp[20], a ketomethylene tripeptide analogue[21] and ACTH related peptides[22]. Kinetic studies of these inhibitors were carried out primarily with HHL used as a substrate. However, both of N^{α}-phosphoryl dipeptides and a ketomethylene tripeptide analogue were reported to behave as competitive inhibitors of ACE when AG I was used as a substrate[20,21]. It is possible that K-26 also inhibits ACE competitively with respect to AG I. Anyway, details on the mechanism of ACE inhibition remain to be clarified. As for the biological activity, K-26, intravenously administered, antagonized completely and specifically the pressor response to AG I in an anesthetized, normotensive rat.

It is interesting that a soil microorganism produces a potent ACE inhibitor like K-26. There may be a good chance of getting clinically useful inhibitors of ACE by the screening of microorganism as well as by chemical synthesis.

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