Purification and Characterization of Endothelin-1 Degradation Activity from Porcine Kidney

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In order to identify the membrane-bound peptidase that is responsible for the degradation of endothelin (ET), an endothelin-1 (ET-1) degradation enzyme was solubilized from membrane fractions of porcine kidney with 1% Triton X-100, and subsequently purified by column chromatographies, *i.e.*, diethylamino-Sepharose ion exchange, gel permeation, Con A Sepharose and hydroxyapatite chromatography. On DEAE-Toyopearl ion exchange column chromatography, the ET degradation enzyme and aminopeptidase were separated, but ET degradation enkephalinase activities were not separable. In order to separate ET degradation enzyme and enkephalinase, the active fractions were loaded on each of the column chromatographies: sephacryl S-200, Con A Sepharose or hydroxyapatite. The ET degradation activities were co-migrated with enkephalinase activities on all of the three chromatographies. In addition, the ET degradation activities were inhibited by thiorphan, phosphoramidon and EDTA, which are known to inhibit enkephalinase. These results suggest that ET degradation activity in the membrane fractions of the kidney is related to enkephalinase and may be involved in the degradation of ET-1 *in vivo*.

Keywords endothelin-1 degradation activity; porcine kidney; enkephalinase; endothelin degradation

Endothelin (ET)-1, produced by endothelial cells, is the most potent vasoconstricting peptide.¹⁾ The production, mechanism of action, and receptors of this unique peptide have been extensively investigated. However, the metabolism or inactivation of ET has not been fully understood. Scicli *et al.* reported that ET-1 was inactivated more potently by the lung and kidney than the other organs, and the degradation activity in the kidney was higher than that in the lung.²⁾ Moreover, Pernow *et al.* also reported that the predominant clearance of ET-1 was shown to take place in the kidney, not in the lung.³⁾

It was reported that enkephalinase cleaved the ET family, but it is not clear that ET is physiologically digested by enkephalinase *in vivo*. Degradation of ET-1 has been shown on the plasma membranes of smooth muscle cells *in vitro*.⁴⁾ Recently, we have shown that ET-1 was digested by neutral peptidase(s) on the membrane fraction of smooth muscle cells.⁵⁾

These notions led us to identify the ET degradation activity on the membrane fraction. In order to clarify the degradation of ET under physiological conditions, we have investigated the degradation of ET in comparison to enkephalinase. Partial purification and characterization of ET degradation enzyme from the kidney is described in this paper.

MATERIALS AND METHODS

Materials were obtained from the following commercial sources and used according to the instructions given by the respective suppliers: μ Bondasphere C₁₈ column from Nihon Millipore, Ltd. (Tokyo, Japan); DEAE-Toyopearl from Tosoh (Tokyo, Japan); thiorphan, captopril and puromycin from Sigma Chemical Co. (St. Louis, U.S.A.); phosphoramidon, E-64, antipain, pepstatin A, Ala-4-methyl-coumaryl-7-amide (Ala-MCA) and ET-1 from

Peptide Institute, Inc. (Osaka, Japan); Wistar rats from Japan SLC, Inc. (Shizuoka, Japan); (3-[¹²⁵I]iodotyrosyl¹³)ET-1 (¹²⁵I-ET-1) from Amersham (Tokyo, Japan); [¹²⁵I]Tyr¹,Leu⁵-enkephalin (¹²⁵I-EK) from NEN (Tokyo, Japan).

Preparation of a Membrane Fraction from Porcine Kidney A porcine kidney (100 g) was minced and homogenized with a Waring blender and a polytron homogenizer in 5 volumes of 20 mM Tris-HCl buffer (pH 7.4, buffer A) containing 0.25 M sucrose. After centrifugation at 3000 × *g* for 20 min, the supernatant was further centrifuged at 100000 × *g* for 1 h. The pellet was washed with the same buffer twice. The pellet was stirred in 100 ml of buffer A containing 0.1% Triton X-100 for 18 h. After centrifugation at 100000 × *g* for 1 h, the pellet was resuspended in 100 ml of buffer A containing 1% Triton X-100 and stirred for 18 h. The extract was obtained by centrifugation at 100000 × *g* for 1 h. All centrifugations and extractions were performed at 4°C.

Assay for ET-1 Degradation Activity by Membrane Fraction from Porcine Kidney Ten microliters of crude enzyme fraction were added to 90 μ l of ET-1 (100 ng/ml) in 0.1 M Tris-HCl buffer (pH 7.4) containing 5% bovine serum albumin (BSA) and 0.5% Triton X-100, and the mixture was incubated for 30 min at 37°C. The reaction was stopped by boiling for 5 min at 100°C. Then, residual ET-1 was measured by sandwich enzyme-linked immunosolvent assay (ELISA), which was carried out as described previously,⁶⁾ using ET-MA51 coated microtiter plates.

Assay for Enkephalinase Activity Five microliters of crude enzyme fraction was added to 10 μ l of 25 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) containing 0.05% Tween 20, 2 mM puromycin and 10 mM captopril, and incubated at 37°C. Five minutes later, 5 μ l of substrate solution (final concentration: 10 nmol/ml EK and about 100000 cpm ¹²⁵I-EK) was added

to the mixture and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 5 μ l of 5 M acetic acid. Three microliters of the reacted mixture was spotted on TLC, and 125 I-Tyr-Gly-Gly, the hydrolyzed product by enkephalinase, was separated from 125 I-EK by TLC on silica gel plates in a mixture of 1-butanol-acetic acid-water (4:1:1) as previously described.⁷⁾ The radioactivities of 125 I-EK and 125 I-Tyr-Gly-Gly were analyzed with an image analyzer system, BAS2000 (Fuji).

Assay for Aminopeptidase Activity Five microliters of crude enzyme fraction and 5 μ l of 2 mM Ala-MCA was added to 90 μ l of 50 mM PIPES buffer (pH 7.4) and incubated for 10 min at 37 °C. The reaction was stopped by the addition of 50 μ l of 5 M acetic acid. The fluorescence of 7-amino-4-methyl-coumarin (AMC), the product by aminopeptidase, was detected with a Fluorescence concentration analyzer (Baxter) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

DEAE-Toyopearl Column Chromatography The extracted solution was loaded on a DEAE-Toyopearl column (5 $\times$ 30 cm) equilibrated with buffer A containing 0.1%

Triton X-100 at a flow rate of 4 ml/min. After the column was washed with 3 l of the same buffer, the column was eluted with 4 l of a linear gradient of NaCl, from 0 to 0.3 M in the same buffer. The fractions which showed ET degradation activity were pooled and concentrated to 30 ml by ultrafiltration using a YM-10 membrane. The protein content was measured by a modified Lowry method.⁸⁾

Effects of Inhibitors To determine the effects of inhibitors, each inhibitor was added to the solution, including partially purified ET degradation enzyme by DEAE ion exchange chromatography. Enzyme inhibitors used were phosphoramidon, thiorphan, EDTA, E-64, captopril, puromycin, antipain, and pepstatin A.

RESULTS

The ET degradation enzyme was solubilized with 1% Triton X-100 from membrane fractions of a porcine kidney. 71.9% of the ET degradation activities were recovered from the membrane fractions. The total activity increased to 247% after the solubilization of the membrane

TABLE I. Partial Purification of ET Degradation Enzyme from Porcine Kidney

Step	ET degradation activity				Aminopeptidase activity
	Total protein (mg)	Total activity (nmol/30 min)	Yield (%)	Purification factor	Residual activity (%)
Starting material (3000 g supernatant)	15070	1.10	100	1	100
Membrane fraction	4540	0.79	71.9	2.4	78.9
1% Triton X-100	ND	2.72	247 ^{a)}		43.0
DEAE-Toyopearl	59	3.05	276 ^{a)}	705	0.5

a) We speculated in Results. ND: Not detectable.

Fig. 1. Profile of DEAE-Toyopearl Ion Exchange Chromatography

Protein (□), aminopeptidase activity (●), ET degradation activity (○) and enkephalinase activity (△) were determined.

fractions with Triton X-100 (Table I). The ET degradation activities in the Triton X-100-solubilized fraction were not reliably assessed by the enzyme-linked immunosolvent assay for ET-1, because the membrane fraction might contain proteins and membranes which likely interfere with degradation activities of ET-1.

The Triton X-100-solubilized fraction was applied to a DEAE ion exchange column. After elution with a linear gradient from 0–0.3 M NaCl, each fraction was assayed for enkephalinase, aminopeptidase and ET degradation activities. Enkephalinase and ET degradation activities were co-eluted on a DEAE ion exchange chromatography, while aminopeptidase activities were separable from ET degradation activities (Fig. 1). The active fractions were pooled, concentrated, and loaded on a column chromatography of sephacryl S-200, Con A Sepharose, or hydroxyapatite. Enkephalinase and ET degradation activities were co-migrated on each of the three column chromatographies (data not shown).

Table II shows the effects of various inhibitors on the

TABLE II. Effect of Inhibitors on the Degradation of ET and Enkephalin

Inhibitor	Final concentration (μ M)	ET degradation activity % of inhibition	Enkephalinase activity % of inhibition
Phosphoramidon	100	108	100
	10	110	100
	1	102	100
Thiorphan	100	100	100
	10	97	98
	1	100	98
EDTA	10000	—	99
	1000	97	86
	100	10	25
	10	2	—
E-64	100	4	29
Captopril	100	6	—
Puromycin	100	0	—
Antipain	100	0	27
Pepstatin A	100	7	47

activities of the partially purified ET degradation enzyme. ET degradation activities were inhibited by phosphoramidon, thiorphan and EDTA, suggesting that it was a metalloprotease. The ET degradation enzyme activities were not inhibited by E-64, captopril, puromycin, antipain or pepstatin A, while enkephalinase activities were partially inhibited by E-64, antipain and pepstatin A.

DISCUSSION

We described the partial purification and characterization of ET degradation enzyme from porcine kidney. ET degradation enzyme and aminopeptidase activities were separable using DEAE ion exchange column chromatography. However, ET degradation activity was co-eluted with enkephalinase, which had been shown to digest ET-1 as well as enkephalin.^{9,10} In attempts to separate ET degradation activity from enkephalinase, we used three different column chromatographies of sephacryl S-200, Con A Sepharose and hydroxyapatite, which was used for the purification of enkephalinase by Malfroy *et al.*¹¹ ET degradation activities co-migrated with enkephalinase activities in each of the three column chromatographies. These results suggest that the ET degradation activity from porcine kidney is not separable from enkephalinase.

The effects of various proteinase inhibitors on ET degradation activities were examined. Phosphoramidon, thiorphan and EDTA, which are known to be enkephalinase inhibitors, also inhibited the ET degradation activities as well as enkephalinase activities (Table II). While ET degradation activities were not inhibited by E-64, antipain or pepstatin A, enkephalinase activities were partially inhibited by these inhibitors. This result might be attributable to contaminating proteinases that digested enkephalin but not endothelin.

To see whether ET degradation activity from porcine kidney is related to enkephalinase, ET degradation enzymes were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Active fractions, pooled and concentrated, were subjected to 10% SDS–PAGE under non-reducing conditions. After elec-

Fig. 2. ET Degradation Activity (A) and Enkephalinase Activity (B) on PVDF Membrane after Enzymes were Transferred from SDS–PAGE

The membrane was cut every three millimeters. Lane 1 contained molecular weight markers. A part of the PVDF membrane was stained with Coomassie Brilliant blue R250 (lane 2).

trophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was cut into slices, and both the enkephalinase and ET degradation activities of each slice were examined. Both activities were identified at the position of 96 kDa, which is known to be the molecular weight of enkephalinase (Fig. 2). Moreover, to identify the enkephalinase on SDS-PAGE, rabbit polyclonal antibodies were raised against the peptide fragment (194—222) of porcine enkephalinase. Dimer positions other than the main position of 96 kDa were recognized by Western blotting using an antibody which reacted with denatured enkephalinase but not with intact enkephalinase.

These data support the idea that ET degradation activity from porcine kidney is related to enkephalinase, and that enkephalinase may play an important role in the degradation of ET-1 *in vivo*. Further purification studies are required to confirm the identity of ET degradation enzyme and enkephalinase.

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