

Purification of a novel endothelin-converting enzyme specific for big endothelin-3

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Abstract Endothelin-3 (ET-3), a potent vasoactive peptide, is considered to be produced from big ET-3 by endothelin-converting enzyme (ECE) like the other members of the endothelin family (ET-1 and ET-2). We purified a novel ECE from bovine iris microsomes. The purified enzyme, a 140 kDa protein by SDS-PAGE analysis, converted big ET-3 to ET-3 but not big ET-1, with a K_m value of 0.14 μ M for big ET-3. The conversion to ET-3 was confirmed with sandwich EIA by monoclonal antibodies, the elution profile of HPLC, and intracellular calcium mobilization in CHO-K1 cells expressing recombinant human ET_B receptors. The conversion activity was inhibited by an inhibitor of neutral endopeptidase 24.11 (NEP) phosphoramidon. These results show that ECE-3 purified from bovine iris is a novel metalloprotease totally different from ECE-1 or ECE-2, in that the enzyme is highly specific for big ET-3.

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Key words: Endothelin-3; Endothelin-converting enzyme; Enzyme purification; Bovine iris

1. Introduction

Endothelin-1 (ET-1), originally isolated from the conditioned medium of cultured porcine aortic endothelial cells, is a potent vasoconstrictor with 21 amino acid residues [1]. The endothelin family consists of three isopeptides: ET-1, ET-2 and ET-3 [2]. In the first step of biosynthesis of ET-1, a large precursor designated preproET-1 is formed. After enzymatic removal of a signal peptide [1,3,4], proET-1 is cleaved by a furin-like protease which belongs to the calcium-dependent serine endoproteases on the C-terminal side of the sequence Arg-Ser-Lys-Arg, leading to formation of big ET-1 [5–8]. Big ET-1 is finally cleaved at the Trp²¹-Val²² bond to form mature ET-1. This reaction is catalyzed by an enzyme called endothelin-converting enzyme (ECE). On the other hand, the precise biosynthetic pathway of ET-3 is not firmly established but is considered to be similar to that of ET-1, based on the following observations. (1) The amino acid sequence of preproET-3 predicted from cloned cDNA has the consensus sequence Arg-X-Lys/Arg-Arg [2] for furin-like enzymes. (2) The big ET-3-like peptide has been identified immunologically in tissues that

produce mature ET-3 [9–11]. (3) ET-3 is produced by incubating big ET-3 with the membrane fraction from bovine endothelial cells [12].

So far cDNAs for two types of ECE have been cloned from rat lung and adrenal cortex, and designated ECE-1 [13–16] and ECE-2 [17], respectively. The predicted amino acid sequence of ECE-1 is similar to that of ECE-2 with an overall identity of 59% [17]. These enzymes are metalloproteases with a zinc-binding motif [13–16]. The enzyme activities are inhibited by phosphoramidon, an inhibitor of neutral endopeptidase 24.11 (NEP), but not thiorphan, another inhibitor of NEP [18–20]. Both ECE-1 and ECE-2 show a high substrate specificity for big ET-1 but not for big ET-2 and big ET-3, although the optimal pHs are slightly different between these two enzymes [17].

In several tissues like the eyeball and some brain regions, the expression of preproET-3 determined by Northern analysis and in situ hybridization was reported to be higher than that of preproET-1 or preproET-2 [21–25], with the highest level in the eyeballs. However, no enzyme specific for big ET-3 has yet been purified. These results encouraged us to purify a novel ECE specific for big ET-3, namely, ECE-3. In this study, we report the purification of ECE-3 from bovine iris and some properties of the purified enzyme.

2. Materials and methods

2.1. Materials

Chemicals were purchased from the following sources: human ET-1, ET-3, big ET-1 and big ET-3 from Peptide Institute Inc. (Osaka, Japan); blue B-agarose from Millipore (Massachusetts, USA); peanut agglutinin (PNA) agarose and wheat germ agglutinin (WGA) agarose from Seikagaku Co. (Tokyo, Japan); a Hiload 26/60 Superdex 200 pg column, a HiTrap Chelating column (1 ml), and a Resource Q column (1 ml) from Amersham Pharmacia Biotech (Uppsala, Sweden); and a Cosmosil 5C18-AR reverse phase column (4.6 mm $\times$ 250 mm) from Nacalai Tesque (Kyoto, Japan), fura-2 acetoxymethyl ester (fura-2/AM) from Dojindo Laboratories (Kumamoto, Japan).

2.2. Solubilization of the bovine iris microsome

Fresh bovine eyeballs were obtained from a local slaughterhouse. Irises were isolated from the surrounding tissues and stored until use in phosphate-buffered saline (PBS) at -80°C . Irises from 200 eyeballs were homogenized in 100 ml of PBS using a Polytron homogenizer at 4°C . All subsequent procedures were carried out at 4°C unless otherwise stated. The homogenates were centrifuged at $1100\times g$ for 15 min, and the resulting supernatant was centrifuged at $140\,000\times g$ for 20 min. For washing, the precipitated microsomes were resuspended in 100 ml of PBS using an ultrasonic disruptor and centrifuged again. This procedure was repeated once more and finally the pellet was resuspended in 100 ml of the solubilizing buffer (10 mM Tris-HCl, pH 7.5, 0.1% lubrol PX). After gentle stirring for 60 min, the sample was centrifuged at $140\,000\times g$ for 20 min, and the resulting super-

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Abbreviations: ET, endothelin; ECE, endothelin-converting enzyme; WGA, wheat germ agglutinin; PNA, peanut agglutinin; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; EIA, enzyme immunoassay; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary

natant was used as a starting material for the subsequent purification steps.

2.3. Purification of ECE-3

Solid sodium chloride was added to the solubilized supernatant to a final concentration of 200 mM. The supernatant was mixed with 10 ml of blue B-agarose equilibrated with buffer A (10 mM Tris-HCl, 200 mM NaCl, 0.1% lubrol PX; pH 7.5) and the mixture was incubated for 50 min. After the agarose had settled (~15 min), the clear supernatant was filtered through 0.2 µm filters of polystyrene, and loaded onto six PNA agarose columns (0.8×4 cm) equilibrated with buffer A. The flow-through fractions were collected and applied onto six WGA agarose columns (0.8×4 cm) equilibrated with buffer A. After washing with 10 ml of buffer B (10 mM Tris-HCl, 1 M NaCl, 0.1% lubrol PX; pH 7.5), the enzyme activity was eluted with 5.5 ml of buffer A containing 50 mg/ml *N*-acetyl-D-glucosamine. The eluate was pooled and concentrated to a volume of approximately 2 ml with a Centriprep-30 concentrator (Millipore). The concentrated eluate was applied onto a Hiload 26/60 Superdex 200 pg column equilibrated with buffer A, and gel filtration was carried out using an Amersham Pharmacia FPLC instrument (Controller LCC-500 Plus equipped with Pump P-500). ECE activity was eluted with buffer A at a flow rate of 1 ml/min and the absorbance of the outflow was monitored at 280 nm. Two-ml fractions were collected, and the activity in each fraction was measured. The fractions with ECE-3 activity were pooled and solid sodium chloride was added to them to a final concentration of 1 M. The pooled fractions were applied onto a HiTrap Chelating column, the matrix of which was charged with zinc ions using 0.1 M ZnSO₄ and subsequently equilibrated with buffer B. The ECE activity was recovered in the pass-through fraction.

2.4. Assay for ECE activity

The standard reaction mixture contained 50 µl of purified ECE-3, 0.5 µM big ETs as a substrate, and 250 µl of ECE reaction buffer (100 mM Tris-HCl, 2 µM ZnCl₂, 1 M NaCl, 1 mM CaCl₂; pH 7.0). The mixture was incubated at 37°C for 1 h and the reaction was stopped by boiling for 5 min. The amount of the produced ETs was measured in triplicate by sandwich enzyme immunoassay (EIA) as described [22,26].

2.5. Analysis of purified ECE-3 by FPLC

The salt concentration of the active fraction from a HiTrap chelating column was reduced to approximately 1 mM by dilution with the solubilizing buffer and subsequent ultrafiltration with a Centriprep-30 concentrator. In this manner, 500 µg of the resulting sample was finally concentrated to approximately 1 ml. It was applied onto a Resource Q column equilibrated with buffer B and eluted with a 25-ml linear gradient of 0–1 M NaCl in buffer B at a flow rate of 0.5 ml/min on the FPLC instrument. 25-µl aliquots from each 1-ml fraction were assayed for ECE-3. Other 10-µl aliquots were analyzed with SDS-PAGE for estimation of the amount of the ECE-3 protein.

2.6. Measurement of the intracellular free calcium concentration ([Ca²⁺]_i) in CHO-K1 cells transfected with cDNA for human recombinant ET_B receptor

Six microgram of purified ECE-3 was incubated at 37°C for 2 h in 300 µl of ECE reaction buffer in the presence of 0.5 µM big ET-3, and the reaction was stopped by adding 3 µl of 0.5 M EDTA. To remove the detergent and other proteins from the product, 200 µl of the reaction mixture was loaded onto a Cosmosil 5C18-AR reverse phase column (4.6 mm×250 mm) connected to HPLC (L-6200 intelligent

pump equipped with L-4250 UV-VIS detector; Hitachi, Japan) and the peptides were eluted with a linear gradient of acetonitrile 0–20% for 5 min, 20–35% for 10 min and 35–40% for 15 min in 0.1% trifluoroacetic acid in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The eluate was monitored at A₂₁₅ and 1-ml fractions were collected. Under this condition, big ET-3 and ET-3 were eluted at retention times of 21.2 and 23.5 min, respectively. The fraction corresponding to the retention time of ET-3 was lyophilized and dissolved in 10 µl of 0.1% acetic acid for administration to CHO-K1 cells. The absolute amount of ET-3 was assayed by sandwich EIA.

For measurement of [Ca²⁺]_i, CHO-K1 cells that stably expressed human ET_B receptors were incubated under reduced light with 5 µM fura-2/AM in Ca²⁺-free Krebs-HEPES solution (140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 1 mM glucose; pH 7.4) for 40 min at 37°C as described [27–29]. After washing with Ca²⁺-free Krebs-HEPES solution, the cells were resuspended in Ca²⁺-free Krebs-HEPES solution at a density of approximately 2×10⁷ cells/ml, and 0.5-ml aliquots were used for measurement of fluorescence with two excitation wavelengths at 340 nm and 380 nm and with an emission wavelength at 500 nm by a CAF-110 intracellular ion analyzer (JASCO, Tokyo, Japan). CaCl₂ (final concentration; 2 mM) was added to the cell suspension immediately before measurement of [Ca²⁺]_i. The [Ca²⁺]_i was calculated from the ratio of fluorescence intensities as described [30].

3. Results

3.1. Distribution of ECE in bovine eyeball

To examine the distribution of the converting activity in bovine eyeballs, the eyeballs were divided into the retina, choroid and iris, and ECE activities in these tissues were assayed (Fig. 1). In the retina and choroid, the conversion activity of big ET-1 to ET-1 was higher than that of big ET-3 to ET-3, whereas both enzyme activities were comparable in the iris. In addition, the conversion activity of big ET-3 was highest in the iris. Therefore, we decided to use the iris for purification of ECE-3.

3.2. Purification of bovine iris ECE-3

The data on purification are summarized in Table 1. The supernatant after solubilization of microsomes was used as a starting material.

Characteristically, the enzyme activity was increased after incubation of the preparation with the blue B-agarose. That is, the enzyme activity which was recovered in the supernatant fraction of the blue B-agarose was increased to 270% of the total activity in the starting material. When the enzyme activity remaining bound to blue B-agarose was eluted and assayed, it was 130% of the activity in the starting material. The recovered activity, amounting to 400% in total, suggests that an inhibitor of the enzyme or a protease capable of inactivating the enzyme was removed at this step. On the other hand, the conversion activity of big ET-1 to ET-1 was unchanged following the incubation with blue B-agarose: about

Table 1
Summary of ECE-3 purification

Fraction	Protein (mg)	Total activity (pmol/h)	Specific activity (pmol/h/mg protein)	Yield (%)	Purification (fold)
Supernatant ^a	69.4	28.3	0.41	100	1
Blue B-agarose (unbound fraction)	46.9	76.2	1.63	270	4.0
PNA agarose (pass-through fraction)	9.98	72	7.21	255	17.7
WGA agarose (eluate)	2.07	77.7	37.5	275	92.1
Hiload 26/60 superdex 200 pg	0.32	45.9	142	162	349
Zinc-chelating Sepharose (pass-through fraction)	0.2	53.2	266	188	653

^aThis sample was prepared from bovine iris of 200 eyeballs.

This purification scheme was replicated four times. Data are from a typical experiment.

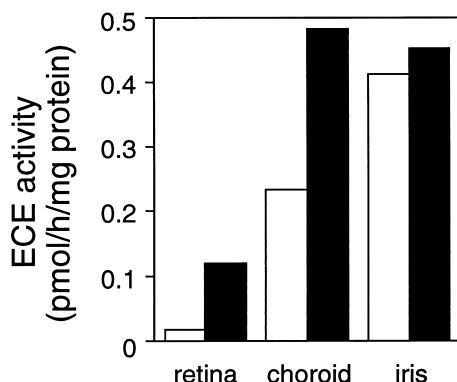


Fig. 1. Distribution of the conversion activity of big ET-1 and big ET-3 in various tissues of bovine eyeball. The eyeballs were divided into the retina, choroid and iris. After homogenization of each tissue, the solubilized microsomes were prepared and the conversion activity of big ET-1 and big ET-3 in each preparation was assayed as described in Section 2. For assays, the microsomal fractions containing 1 mg of protein (bovine retina, choroid and iris) were incubated in the presence of 0.5 μ M big ET-1 or big ET-3 in ECE reaction buffer for 1 h at 37°C, respectively. The produced ET-1 (closed column) and ET-3 (open column) were measured using sandwich EIA.

half (52%) of the total activity in the starting material was recovered in the supernatant fraction and the remaining half was bound to the blue B-agarose (48%). Therefore, the supernatant fraction was used for further purification of ECE-3. The supernatant fraction was subsequently applied onto a PNA agarose column and again the ECE-3 activity was recovered in the pass-through fraction with a 4.4-fold purification

and 94% recovery. WGA agarose gave the best results among the affinity columns tested, providing a 5.2-fold purification. After gel filtration by FPLC, ECE-3 activity was reproducibly recovered in the fractions with retention times of 130–142 min and a 3.8-fold purification was obtained. Finally, the fractions from FPLC were pooled and applied onto a HiTrap chelating column: ECE-3 activity was recovered with a 1.9-fold purification. The overall purification of ECE-3 activity was approximately 653-fold from the starting material with an apparent recovery of 188%. When the fractions from each purification step were examined by SDS-PAGE (Fig. 2), the enzyme was found to be purified to homogeneity as a single band at 140 kDa after the HiTrap chelating column procedure. Furthermore, to confirm that the enzyme activity is derived from the purified protein, anion-exchange chromatography was performed. The elution pattern of the enzyme activity in each fraction was found to be correlated with that of the protein (Fig. 3).

3.3. Basic properties of ECE-3

The conversion of big ET-3 to ET-3 increased linearly with time, but no plateau was reached within the observation time (Fig. 4a). ECE-3 activity increased with the increases in big ET-3 and reached a maximal activity of 3.5 pmol/min/mg protein at concentrations higher than 2×10^{-7} M. In contrast, the conversion activity for big ET-1 was not detected up to a concentration of 2×10^{-6} M (Fig. 4b). Higher concentrations of big ET-1 cannot be tested because of its solubility. Lineweaver-Burk double reciprocal plots revealed that the K_m and V_{max} values for big ET-3 were 0.14 μ M and 7.4 pmol/min/mg protein, respectively (Fig. 4c). The optimal pH was around 6.6

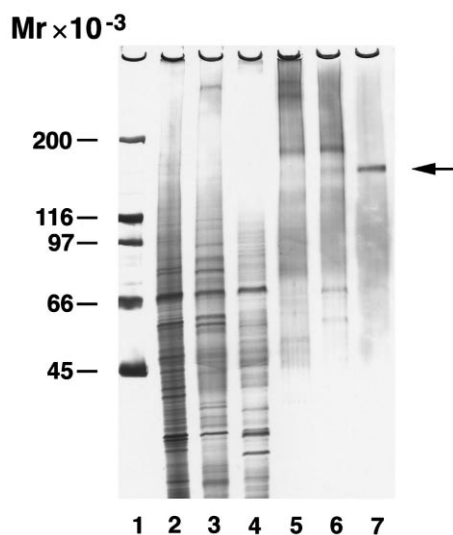


Fig. 2. SDS-PAGE analysis of various fractions obtained during purification of ECE-3. Samples from each purification step were electrophoresed on an SDS-polyacrylamide gel with a 4–20% gradient of polyacrylamide and the protein was visualized with silver staining. Lane 1, molecular weight standards (100 ng of each protein); myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa). Lane 2, 10 μ g of solubilized protein from iris microsomes with 0.1% lubrol PX. Lane 3, 1 μ g of the fraction unbound to blue B-agarose. Lane 4, 1 μ g of the pass-through fraction from PNA agarose. Lane 5, 1 μ g of the eluate from WGA agarose. Lane 6, 500 ng of pooled fraction from Hiload 26/60 Superdex 200 pg. Lane 7, 500 ng of the pass-through fraction from HiTrap chelating column. An arrow indicates purified ECE.

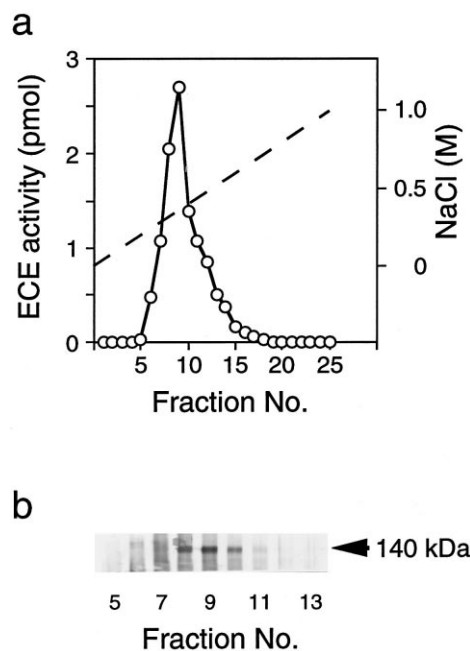


Fig. 3. Analysis of purified ECE by FPLC. After concentration, the purified ECE (the pass-through fraction from HiTrap chelating column) was applied onto the anion-exchange column (Resource Q) connected with FPLC and eluted with a 25-ml linear gradient of 0–1 M NaCl in buffer B at a flow rate of 0.5 ml/min. A 25- μ l aliquot from each 1-ml fraction was used for the assay of enzyme activity (a) and the other for analysis by SDS-PAGE (b). The dashed line indicates the concentration of NaCl in the elution buffer. An arrow indicates the purified ECE.

(Fig. 4d). The enzyme activity was inhibited by phosphoramidon in a concentration-dependent manner, with an apparent IC_{50} value of $0.05 \mu\text{M}$, and complete inhibition was obtained at $10 \mu\text{M}$. In contrast, the enzyme activity was unaffected by thiorphan (Fig. 4e).

3.4. Measurement of $[Ca^{2+}]_i$ in CHO-K1 cells transfected with cDNA for human recombinant ET_B receptor

To confirm that the product of the purified enzyme possesses the same biological activity as ET-3, we examined the effect of the product on $[Ca^{2+}]_i$ in CHO-K1 cells expressing human ET_B receptors. As shown in Fig. 5a, authentic 10^{-9} M ET-3 induced an increase in $[Ca^{2+}]_i$ in the CHO-K1 cells. When the enzyme product isolated as described in Section 2

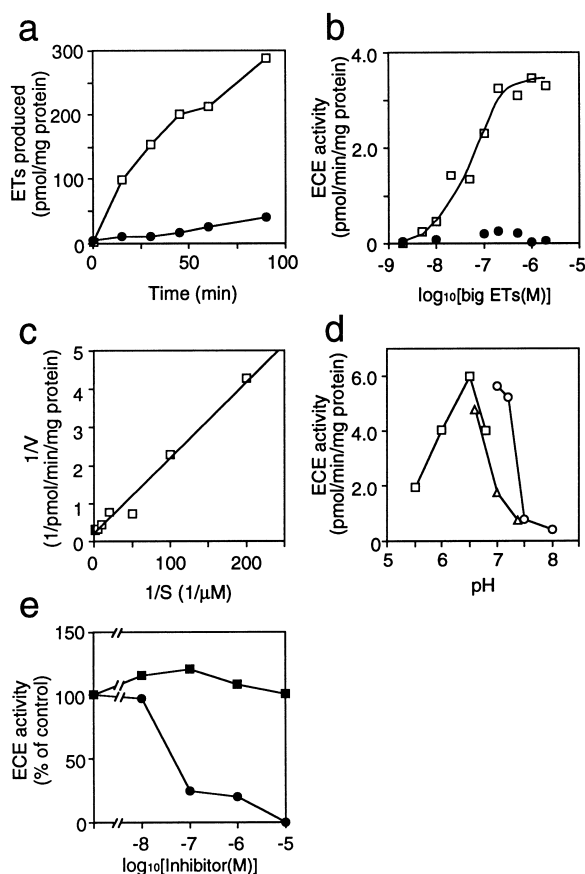


Fig. 4. Basic properties of the purified ECE-3. (a) Time course of ECE reaction. $3 \mu\text{g}$ of the purified ECE was incubated with $0.5 \mu\text{M}$ big ET-3 or $0.5 \mu\text{M}$ big ET-1 in $300 \mu\text{l}$ of ECE reaction buffer at 37°C for the indicated time, and the produced ET-3 (open square) or ET-1 (closed circle) was measured. (b) Effect of substrate concentration on the reaction rate. The assay of ECE activity was performed in the same manner except that the reaction time was 60 min with the concentrations of big ET-3 being varied. Open squares, big ET-3; closed circles, big ET-1. (c) Lineweaver-Burk double reciprocal plots of data on ECE-3 activity in panel b. (d) pH profile of ECE-3 activity. The reaction was performed for 60 min in reaction mixture containing the indicated buffers. That is, 50 mM MES buffer (square), 100 mM sodium phosphate buffer (triangle) and 100 mM Tris buffer (circle) were used for the pH ranges of $5.5\text{--}6.8$, $6.2\text{--}7.4$ and $7.0\text{--}8.0$, respectively. (e) Effect of phosphoramidon and thiorphan on ECE-3 activity. The reaction was essentially similar except that the reaction mixture contained the indicated concentrations of phosphoramidon (closed circle) or thiorphan (closed square).

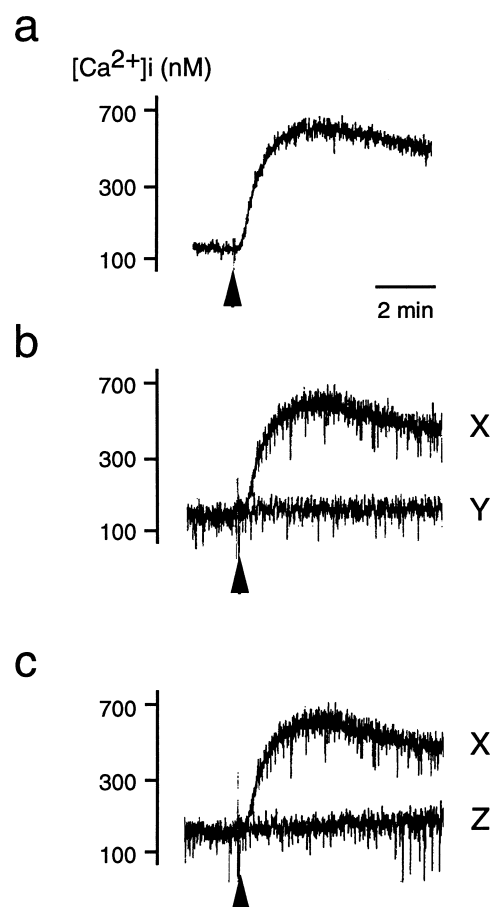


Fig. 5. Effect of the purified ECE-3 product on $[Ca^{2+}]_i$ in CHO-K1 cells expressing human ET_B receptors. $6 \mu\text{g}$ of the purified ECE ($50 \mu\text{l}$) was incubated at 37°C in $250 \mu\text{l}$ of ECE reaction buffer in the presence of $0.5 \mu\text{M}$ big ET-3 for 2 h (b and c; X) or 0 h (b; Y). $200 \mu\text{l}$ of the reaction mixture was applied onto a Cosmosil 5C18-AR reverse phase column and the peptides were eluted with a linear gradient of acetonitrile. The fraction corresponding to the retention time of ET-3 was collected, lyophilized and dissolved in $10 \mu\text{l}$ of 0.1% acetic acid. $1 \mu\text{l}$ of the solution was added to a 0.5-ml suspension of CHO-K1 cells expressing human ET_B receptors in the absence (b) or presence (c; Z) of $1 \mu\text{M}$ BQ788 (an antagonist of ET_B receptor). (a) Effect of authentic 10^{-9} M ET-3 on $[Ca^{2+}]_i$. An arrow indicates the addition of authentic ET-3 or the purified enzyme products.

was added to the cells, it induced an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ induced by the enzyme product containing 0.22 pmol (concentration: $0.44 \times 10^{-9} \text{ M}$) was comparable to that by 10^{-9} M ET-3. In contrast, no response was observed when the sample was prepared from blank reaction mixtures without either the purified enzyme or the substrate big ET-3, or the reaction mixture without incubation (Fig. 5b). The response of transfected CHO-K1 cells to the enzyme product was abolished by 10^{-6} M BQ788, a specific antagonist for ET_B receptors (Fig. 5c), but not by 10^{-6} M BQ123, a specific antagonist for ET_A receptors (data not shown).

4. Discussion

In the present study, a novel enzyme which produces an ET-3-like substance from big ET-3 was purified to apparent homogeneity from bovine iris.

This enzyme is characterized by a high selectivity for big ET-3 over big ET-1 (Fig. 4), which distinguishes the purified enzyme from ECE-1 or ECE-2 with a reverse order of selectivity [14–17]. That is, the K_m value of the purified enzyme for big ET-3 is low (0.14 μM), whereas conversion of big ET-1 is not detected up to 10 μM .

The product of this enzyme is identified as ET-3 itself or a peptide with properties very close to ET-3 based on the following observations. The enzyme product has (1) the same immunoreactivity as ET-3, (2) the same retention time as ET-3 on HPLC with a reverse phase column, and (3) the same biological activity as ET-3, in that it can raise $[\text{Ca}^{2+}]_i$ in the cells expressing ET_B receptors with a potency comparable to that of authentic ET-3 (Fig. 5). Taken together, these results show that the purified enzyme is a new member of the ECE family, namely ECE-3.

Furthermore, the molecular mass of the purified enzyme appears to be about 140 kDa (Fig. 2), which is slightly larger than those of ECE-1 and ECE-2 (130 kDa) [13,14].

This enzyme seems to be a metalloprotease like other members of the ECE family such as ECE-1 and ECE-2, based on the data that the activity of the purified enzyme is blocked by phosphoramidon but not by thiorphan.

The extent of purification appears to be not so high as that of ECE-1 [31]. This seems to be mainly due to co-localization of other types of ECE like ECE-1 or ECE-2 in bovine iris which can convert big ET-3 to ET-3 as previously described [14,17]. Therefore, the actual fold purification could be higher, unless the starting material did not contain ECE-1 activity.

We are trying to microsequence the purified enzyme and isolate cDNA for ECE-3.

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