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PEPSIN, AN ASPARTIC PROTEASE, CONVERTS PORCINE BIG ENDOTHELIN TO 21-RESIDUE ENDOTHELIN

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SUMMARY: Porcine big endothelin (big ET-39) at 1 nM, a concentration with no influence on contractile activity in isolated rat aorta, induced a slow-onset and sustained contraction by the pre-incubation with pepsin. When the incubation mixture of big ET-39 with pepsin was analyzed by high-performance liquid chromatography on an octadecyl silica column, two major products of pepsin hydrolysis were obtained; their amino acid sequences were identical with those of 21-residue endothelin (ET-21) and a C-terminal peptide of big ET-39, big ET (22-39), respectively. On the other hand, no degradation of ET-21 was observed by pepsin treatment. These results indicate that pepsin specifically cleaves a Trp^{21}-Val^{22} bond in the big ET-39 molecule, producing ET-21 and big ET (22-39). Thus, the possibility that pepsin-like aspartic protease may participate in the conversion of big ET-39 to ET-21 in vivo warrants further attention.

Endothelin (ET), isolated from culture medium of porcine aortic endothelial cells (EC), is a potent vasoconstrictor peptide consisting of 21 amino acid residues with two intramolecular disulfide bonds (1). Based on the amino acid sequence of prepro ET deduced from the nucleotide sequence of porcine ET cDNA, Yanagisawa et al. (1) proposed that mature ET (ET-21) is produced by a putative "ET-converting enzyme", through an unusual proteolytic processing between Trp^{21} and Val^{22} of an intermediate form consisting of 39 amino acid residues, termed big ET-39. There is accumulating evidence that big ET-39, in addition to ET-21, is actually biosynthesized and secreted by vascular EC (2,3).

Yanagisawa et al. (1) also suggested that an endopeptidase with chymotrypsin-like protease specificity is presumably involved in the proteolytic processing between Trp^{21} and Val^{22} of big ET-39. McMahon et al. (4) reported data showing that chymotrypsin cleaves not only the Trp^{21}-Val^{22} bond, but also the Tyr^{31}-Gly^{32} bond in big ET molecule. If big ET-39 is processed by a chymotrypsin-like endopeptidase for the production of ET-21, two peptide fragments corresponding to big ET (22-31) and big ET (32-39) would be produced and released by vascular EC, as is ET-21. However, most recent studies demonstrated the presence of big ET (22-39), but not big ET (22-31) or

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big ET (32-39), in culture supernatant of porcine aortic EC (3). This suggests that the processing of big ET-39 to ET-21 occurs only between Trp^{21} and Val^{22}, and prompted us to explore an endopeptidase susceptible to the Trp^{21}-Val^{22} bond.

We have now examined the conversion of big ET-39 to ET-21 by pepsin, an aspartic protease which hydrolyzes peptide bonds at the C-terminal side of aromatic amino acids, such as Trp, Tyr and Phe, and obtained evidence that pepsin specifically cleaves a Trp^{21}-Val^{22} bond of big ET-39, producing ET-21 and big ET (22-39).

MATERIALS AND METHODS

Chemicals: Synthetic big ET-39 and ET-21 were purchased from Peptide Institute Inc. (Osaka, Japan), dissolved with sterilized water at a concentration of 0.1 mM and stored at 4° C until use. Pepsin (porcine stomach mucosa, 3900 units per mg protein) was obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A.), freshly dissolved and diluted with 50 mM citric acid (pH 2.3) before use.

Pepsin Treatment: Big ET-39 (4.4 µg, 1 nmol) and ET-21 (2.5 µg, 1 nmol) were treated with pepsin (1 µg) in a final volume of 0.1 ml as follows. Fifty µl of pepsin solution (20 µg/ml) was prewarmed with 40 µl of 50 mM citric acid (pH 2.3) at 37° C for 5 min, and then 10 µl of big ET-39 or ET-21 (100 nmol/ml) was added. After incubation for 1-60 mins, each reaction mixture was placed in an ice-bath and immediately subjected to high-performance liquid chromatography (HPLC). The control was determined by the addition of 50 mM citric acid instead of pepsin solution. For bioassay, the mixture was neutralized by the addition of 0.9 ml of 0.1 M Tris-HCl buffer (pH 8.0) and then diluted with Krebs-Ringer bicarbonate solution (118.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose).

Bioassay: The vasoconstrictor activitiy of big ET-39 treated with or without pepsin was assayed by measuring isometric contractile responses of helically cut strips of the rat thoracic aorta with the intima denuded mechanically, as described elsewhere (5). The contractile responses were expressed either as a percentage of that to 60 mM KCl or as an absolute change in tension.

High-Performance Liquid Chromatography: Reversed-phase HPLC analyses were performed with a Waters multi-solvent delivery system, a Model 600E and a Model U6K injector coupled to a Model 990J photodiode array detector. The column used was Capcell-Pak $5C_{18}$-SG300 (4.6 x 250 mm) from Shiseido (Tokyo, Japan). Elution was performed by using 0.02% trifluoroacetic acid (TFA) in water (solvent A) and 0.02% TFA in acetonitrile (solvent B). Gradient consisted of a linear gradient from 0 to 35% solvent B in 15 min, followed by isocratic elution at 35% solvent B for 15 min and a linear gradient from 35 to 63% solvent B in 15 min. The flow rate was 0.5 ml/min. Eluates were monitored by absorbance at 215 nm. Under these conditions, big ET-39 and ET-21 were eluted at a retention time of 41 and 43 min, respectively and were completely separated (Fig. 1).

Sequence Analysis: Automated Edman degradation was performed with a gas-phase protein sequencer (Model 477A, Applied Biosystems Inc.). The resulting phenylthiohydantoin (PTH) derivatives were identified by a PTH-analyzer (Model 120A, Applied Biosystems Inc.), linked on line with the gas-phase sequencer.

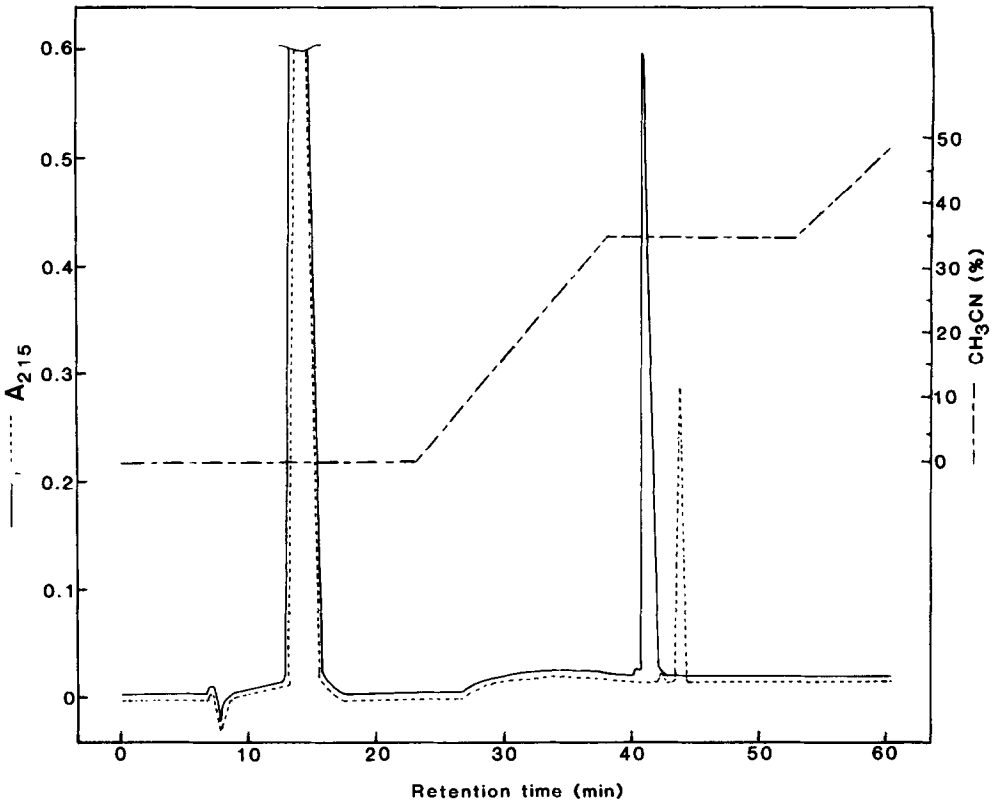

Figure 1. Elution profiles of big ET-39 and ET-21 by HPLC on a Capcell-Pak C_{18} column. Ten µl of each peptide (1 nmol) was added to 90 µl of 50 mM citric acid (pH 2.3) and applied to the column. Details of the elution conditions are given in the text. ——, big ET-39; ----, ET-21.

RESULTS AND DISCUSSION

As shown in Fig. 2, big ET-39 at 1 nM produced no contraction of rat aortic strips, a response in agreement with that obtained using porcine coronary artery strips with the endothelium removed (6). Pepsin alone had no effect on the contractile activity of the aortic strips (data not shown). On the other hand, a slow-onset and sustained contraction of the strips was observed when big ET-39 was incubated with pepsin for various periods then added to the organ bath under the same conditions as for big ET-39 alone. Incubation of big ET-39 with pepsin resulted in a time-related increase in the contraction. The maximal contractile response to big ET-39 incubated with pepsin for 30 min gave 101 ± 8.3% of the 60 mM KCl-induced contraction, the value being almost equal to that obtained with ET-21 at 1 nM, as been reported elsewhere (5). Kimura et al. (6,7) tested the contractile responses to ET related peptides on porcine coronary artery strips and found that ET-21 is the most active form and that the conversion of big ET-39 to ET-21 is essential to generate the physiologically most active form. The present data, taken

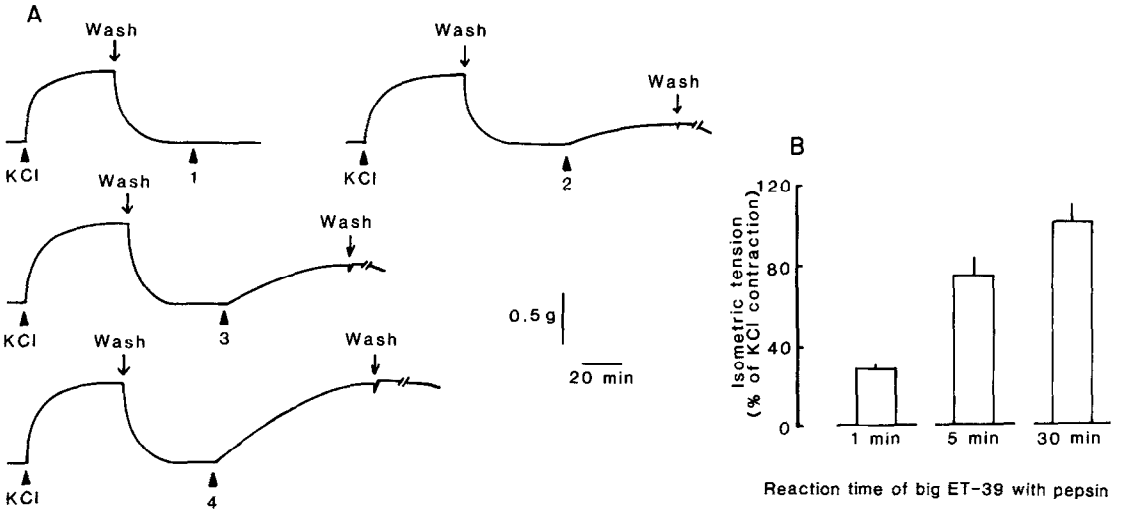

Figure 2. Contraction of rat aortic strips induced by big ET-39, with or without pepsin treatment. A, typical contractile responses to 60 mM KCl, 1 nM big ET-39 alone (1) and big ET-39 treated with pepsin for 1 min (2), 5 min (3) or 30 min (4). The contractile responses are expressed as an absolute change in tension. B, mean contractile responses; columns and bars represent the mean and S.E.M. of 3 separate experiments. The contractile responses are expressed as percentages of the maximum tension increment induced by 60 mM KCl.

together with the structure-activity relationship studies on ET related peptides (6,7), make it reasonable to assume that ET-21 is formed during incubation of big ET-39 with pepsin and that it produces a potent contraction of rat aortic strips.

The above finding suggested to us that with pepsin treatment, big ET-39 converts to ET-21 with the release of a C-terminal peptide consisting of 18 amino acid residues. To obtain supportive evidence, we first isolated peptide fragments formed during the incubation of big ET-39 with pepsin. Figure 3 shows reversed-phase HPLC profiles of big ET-39 and ET-21, with or without pepsin treatment. When big ET-39 was incubated with pepsin for 1 min and then subjected to an octadecyl silica column, three major peaks were detected at retention times of 36, 41 and 43 min, respectively; we tentatively designated them as peak 1, peak 2 and peak 3 in the order of elution. The retention times of peak 2 and peak 3 corresponded to those of big ET-39 and ET-21, respectively. A few minor peaks were also observed between peak 1 and peak 2. Increasing incubation time resulted in a decrease in the area of peak 2 (big ET-39). Peak 2 almost disappeared after the incubation for 30 min. On the contrary, the areas of peak 1, peak 3 and minor peaks increased progressively with increasing incubation time and attained the approximate maximal level after 30 min. No detectable degradation of ET-21 with pepsin treatment was observed even after incubation for 60 min. Furthermore, no peak

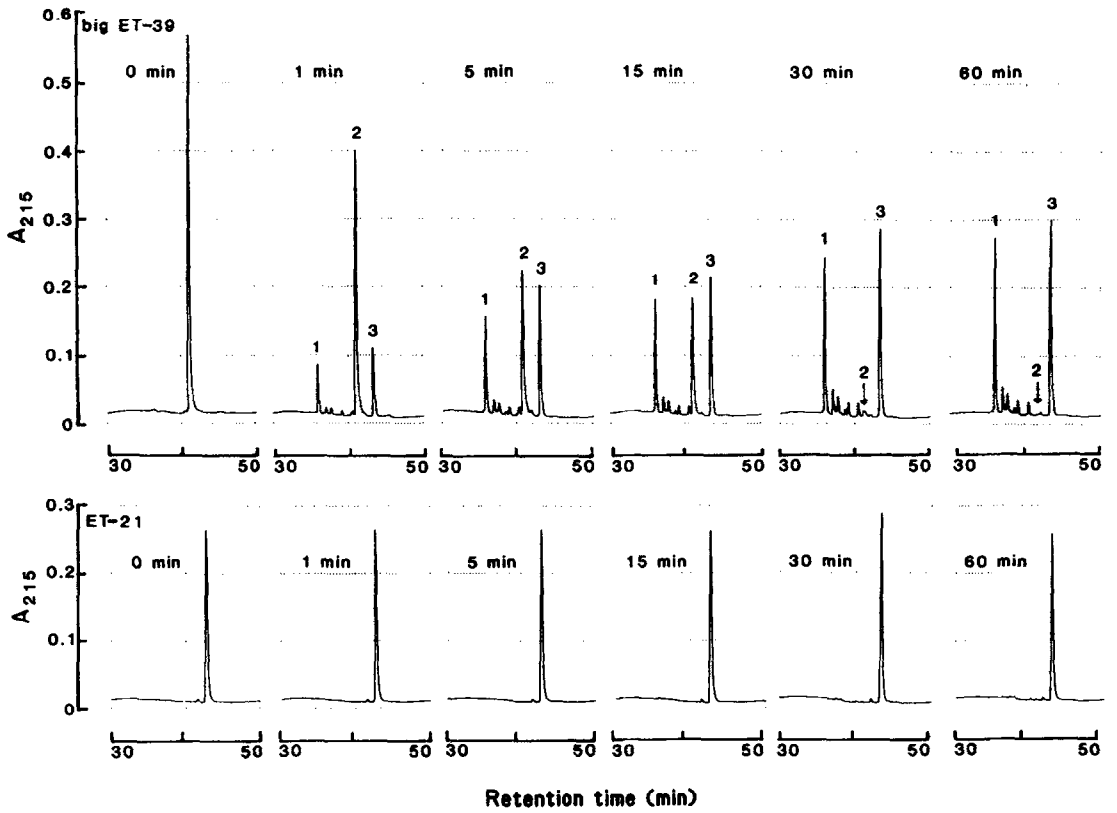

Figure 3. Reversed-phase HPLC profiles of big ET-39 and ET-21, with or without pepsin treatment. Big ET-39 (4.4 μg) or ET-21 (2.5 μg) was incubated with pepsin (1 μg) for various periods of time indicated. Then, each sample was applied to a Capcell-Pak C_{18} column. Elution conditions were the same as for Fig. 1.

was present between a retention time of 30 and 50 min, with HPLC of pepsin alone (data not shown). These results indicate that peaks 1 and 3 are major products of the pepsin hydrolysis of big ET-39 and that pepsin probably degrades part of the newly generated peak 1, thereby producing the small peptide fragments between peak 1 and peak 2.

We next determined the amino acid sequences of peak 1 and peak 3 obtained by HPLC of big ET-39 treated with pepsin for 30 min. As shown in Fig. 4, peak 1 comprised 18 amino acid residues, each of which was detected as a single PTH-amino acid. The sequence corresponded to big ET (22-39). Automated Edman degradation of peak 3 shows that the amino acid sequence was identical with that of ET-21, although no PTH-amino acid derivative could be detected at positions 1, 3, 11 and 15. The unidentified amino acid corresponds to Cys present at the same positions in both the big ET-39 and ET-21 sequences. It is, therefore, conceivable that Cys is conserved at the equivalent position in the peak 3. PTH-Cys was undetectable in the present experiments as we did not use pyridylethylated samples for sequence analysis.

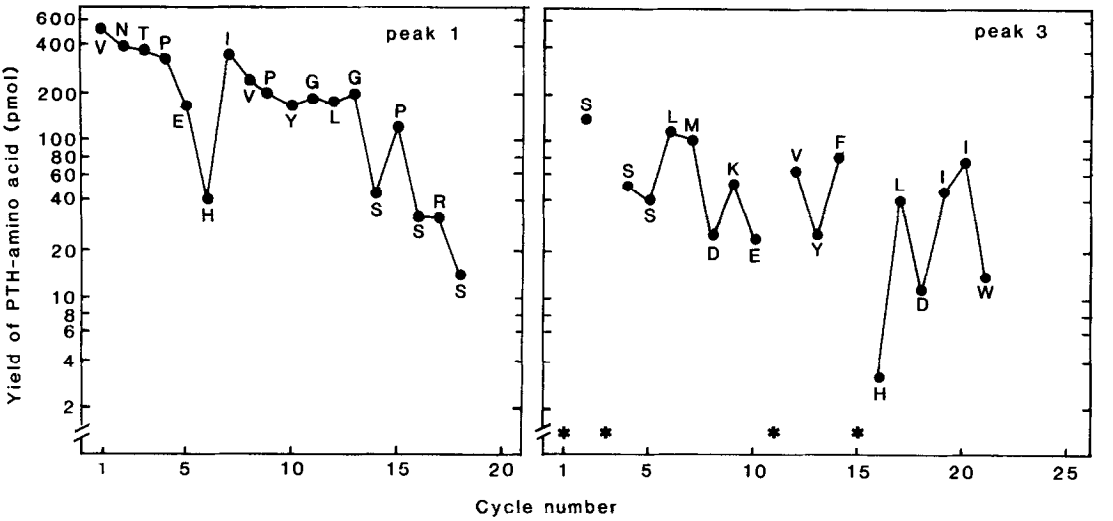

Figure 4. Sequence analyses of cleavage products of big ET-39 by pepsin. Big ET-39 (4.4 μg) was incubated with pepsin (1 μg) for 30 min and applied to a Capcell-Pak C_{18} column. The two major products obtained, peak 1 and peak 3, were sequenced. The PTH-amino acid at each cycle of automated Edman degradation is shown by the one-letter symbol. *, not identified.

The sequence analysis, along with experiments performed by HPLC, strongly indicates that ET-21 and big ET (22-39) are generated from big ET-39 by peptic cleavage between Trp^{21} and Val^{22}.

Big ET-39 and ET-21 are found to be biosynthesized and released by vascular EC (2,3). However, the question remains as to how big ET-39 converts to ET-21 in the EC, since no one has yet characterized an endopeptidase responsible for the conversion. Taking into account our present results, it is possible that a pepsin-like endopeptidase could convert big ET-39 to ET-21 in vascular EC. In fact, we recently obtained data that a pepstatin-sensitive aspartic protease is involved in the conversion of big ET-39 to ET-21 in porcine aortic EC (Matsumura et al., unpublished data).

In conclusion, the present study demonstrated that pepsin specifically cleaves a Trp^{21}-Val^{22} bond of big ET-39 to generate ET-21 with the release of big ET (22-39). Thus, pepsin is a specific tool for investigations related to the production of ET-21, and a pepsin-like aspartic protease may participate in the processing of big ET-39 to ET-21 in vascular EC.

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