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High-yield production of human big endothelin-1 by a combination of chemical modification and proteolysis of a fusion protein in *Escherichia coli*

**Abstract** A protein modification method has been developed for the production of human big endothelin (ET)-1. Production of a large quantity of big ET-1 by the method described here is expected to facilitate future experiments such as X-ray crystallography and nuclear magnetic resonance studies, aimed at understanding the tertiary structure of big ET-1 and its dynamics. The plasmid pETB-50 used for the synthesis carries the gene for a fusion protein consisting of 34-amino acid (aa) residues of an N-terminal portion of β-galactosidase and the 38-aa residues of big ET-1. The fusion protein ETB-50P contains an arginine residue in the big ET-1 portion at its second C-terminal site and three lysine residues including the C-terminal site in the β-galactosidase portion, all of which are susceptible to trypsin. Tryptic digestion of the fusion protein quantitatively produced big ET-1 (1-37), which is depleted in the C-terminal serine. However, a treatment of the fusion protein with 1, 2-cyclohexanedione prior to tryptic digestion gave full-length big ET-1 with Nε,Nδ-(1,2-dihydroxy-cyclohex-1,2-ylene)-arginine. This modification was reversed to the intact arginine residue when the modified big ET-1 was incubated in 0.5 M TRIS-HCl buffer, pH 8.0. Consequently, a combination of such a reversible protein modification and tryptic digestion gave 1.74 mg of recombinant big ET-1 from 2.0 l of culture broth. The procedure described here may be applied to produce other arginine-containing peptides from fusion proteins.

**Introduction**

Endothelin (ET)-1 is an extremely potent and durable vasoconstrictive 21-residue peptide, and has four cysteine residues at positions 1, 3, 11 and 15, which form two intrachain disulfide linkages between 1-15 and 3-11. Human ET-1 is produced from a 212-residue precursor via a 38-amino acid (aa) intermediate called big ET-1 by a specific and unusual proteolytic cleavage between Trp₁ and Val₂ by a putative endothelin-converting enzyme (Yanagisawa et al. 1988; Itoh et al. 1988; Okada et al. 1990). Further studies have proved the existence of two additional related peptides, termed ET-2 and ET-3 (Inoue et al. 1989; Matsumoto et al. 1989). ET isopeptides are involved in numerous biological responses such as constriction of various smooth muscles (Yanagisawa et al. 1988; Advenier et al. 1990), cardiac effects (Ishikawa et al. 1988) and mitogenic action (Takuwa et al. 1989). ETs have also been suggested to possess relevance to hypertension (Saito et al. 1990), renal failure (Tomita et al. 1989), vasospasm (Kurihara et al. 1989), or endotoxic shock (Marel et al. 1989). However, the pathophysiological roles of ETs are still unclear. ET-Converting enzyme inhibitors should be good tools for studying physiological effects and physiological significance of ET. Random oxidation of fully reduced ET-1 results in a mixture of [Cys¹-Cys¹⁵/Cys³-Cys¹¹] form and [Cys¹-Cys¹¹/Cys³-Cys¹⁵] form. In order to selectively obtain the native ET-1, namely [Cys¹-Cys¹⁵/Cys³-Cys¹¹] form, a segment condensation method must be employed using specifically removable protecting groups at the cysteine residues (Kumagaye et al. 1988). If big ET-1 can be easily prepared in large quantities, it will provide a good source for structural studies such as X-ray crystallography and nuclear magnetic resonance (NMR) study, which are expected to lead us to the modeling of ET-converting enzyme inhibitors.

We (Ohashi et al. 1991) have previously described the production of bacterially expressed human big ET-1 using proteolytic cleavage by blood coagulation...
factor Xa (FXa) or trypsin of the fusion protein ETB-42P. This fusion protein consists of 80-aa residues: the 38-aa N-terminal portion of β-galactosidase and the 38-aa residues of big ET-1 are linked by the 4-aa FXa recognition sequence (Ile-Glu-Gly-Arg). However, FXa did not stoichiometrically release big ET-1 from ETB-42P and the conversion rate was only 30%. Tryptic digestion was alternatively tested, since arginine in the FXa sequence could be recognized by trypsin. However, big ET-1 (1–37) depleting the C-terminal serine contaminated in the digest, because of arginine at the 37th residue that was recognizable by trypsin. An extensive digestion predominantly produced big ET-1 (1–37). Even in an optimal condition of the partial trypsic digestion of ETB-42P, the yield of big ET-1 (1–38) was only 14%. Arginylendopeptidase cleaved big ET-1 between the Arg37-Ser38 bond, and lysylendopeptidase cleaved ET-1 between the Lys9-Glu10 bond (Ohashi and Hata, unpublished data), therefore both enzymes were not suitable for production of big ET-1 by proteolysis of fusion proteins synthesized in Escherichia coli.

1. 2-Cyclohexanedione (CHD) is a reagent used to modify the guanidino group of the arginine residue to N2,N8-(1,2-dihydroxy-cyclohex-1,2-ylene)-arginine (DHCH-arginine) (Toi et al. 1967; Patthy and Smith 1975a,b). Since the modified arginine is resistant to trypsic digestion, CHD is commonly used prior to trypsic digestion in order to restrict the specificity of the protease to lysine residues. It is advantageous that the DHCH-arginine can be easily reversed to arginine by hydroxylamine treatment (Patthy and Smith 1975a,b).

To improve the yield of full-length big ET-1 (1–38) from fusion proteins by trypsic digestion, we adopted this reversible arginine-modification method using CHD. As ETB-42P disadvantageously contained the arginine residue in the FXa recognition sequence ahead of the big ET-1 portion (Ohashi et al. 1991), we constructed a new plasmid, pETB-50, encoding a lysine residue instead of arginine at the interface of the two peptide domains, in order to prepare a selective cleavage site even after CHD modification. We also describe properties of big ET-1 obtained by such procedures.

**Materials and methods**

**Bacterial strains, plasmid and medium**

Host strains used for plasmid construction or gene expression were E. coli HB101 (Boyer and Roulland-Dussios 1969) and JM109 (Yanisch-Perron et al. 1985). E. coli cells were grown with aeration in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) at 37°C. Ampicillin (50 µg/ml) was added to the medium for the E. coli cells containing recombinant plasmid. The construction and characterization of plasmid pETB-42 was as previously described (Ohashi et al. 1991). Plasmid pBluescriptII containing polynuker cloning sites within the coding region of the β-galactosidase gene (lacZ) was the product of Stratagene.

**Reagents**

Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were obtained from Takara Shuzo (Kyoto, Japan). Trypsin (modified by reduction alkylation) was the product of Promega. Unless otherwise stated, enzyme reactions were performed following the manufacturer’s protocol. CHD was purchased from Wako Pure Chemicals (Osaka, Japan). Authentic ET-1, human big ET-1, chymostatin, and phosphoramidon were purchased from the Peptide Institute (Osaka, Japan).

**Oligonucleotide synthesis**

Oligonucleotide-A (5′-GATCCCCAAATGTCTCGAGCTC-CCTGATGGATAAAGAGTGTGT-3′) containing the codon (AAA) for lysine and the PstI recognition site (5′-CTGCAG-3′), and its complementary oligonucleotide-B (5′-AGACACACTCTTTATCCATCAGGGAGCTGCAGGAGCATFTGG-3′), were chemically synthesized on an Applied Biosystems 380A DNA synthesizer. The annealed oligonucleotide-A and -B formed the cleavage sequences recognizable by restriction enzymes BamHI and AccI at the end sides.

**Plasmid construction**

As shown in Fig. 1, pETB-42 was digested with BamHI and AccI, and the larger BamHI/AccI DNA fragment was purified by the low-melting agarose gel electrophoresis method. Oligonucleotide-A and -B were allowed to anneal after phosphorylation with T4 DNA polynucleotide kinase and ligated with the BamHI/AccI DNA fragment by T4 DNA ligase. The novel plasmid construct, pETB-50, containing the oligonucleotide encoding lysine prior to big ET-1 peptide was confirmed by the existence of PstI recognition site and DNA sequencing.

**Fusion protein purification**

The fusion protein ETB-50P, which formed inclusion bodies in E. coli cells, was isolated by centrifugation from the lysed cells and solubilized in 8 M urea, followed by a DEAE-Toyopearl 650 M column chromatography as previously described in the purification of ETB-42P (Ohashi et al. 1991; Yasufuku et al. 1992).

**Production of big ET-1 by proteolysis of the arginine-modified ETB-50P**

Figure 2 shows the schematics procedure employed in the present study. The purified ETB-50P (6.0 mg, 0.78 µmol) was dissolved in 10 ml freshly prepared 0.25 M borate buffer, pH 9.0, supplemented with 65.3 mg (0.58 mmol) of CHD; the reaction mixture was kept under N2 gas at room temperature to modify arginine residues. After 1.5 h the reaction was stopped by the addition of 10 ml of 30% acetic acid, and subjected to preparative reversed-phase HPLC to isolate the ETB-50P with DHCH-arginine residues (DHCH-50P). The lyophilized DHCH-50P (5.49 mg) was dissolved in 0.1 M borate buffer, pH 8.0, containing chymostatin (1.5 µg/ml) at a concentration of 0.1 mg/ml and hydrolyzed with 330 µg trypsin at 30°C for 2 h. The digestion was stopped by adding acetic acid to 30%, and the reaction mixture was subjected to preparative reversed-phase HPLC to isolate the DHCH-big ET-1. The purified DHCH-big ET-1 (2.52 mg) was dissolved in 1 ml of 8 M urea and 9 ml of 0.56 M TRIS-HCl buffer, pH 8.0, was added to resume intact big ET-1. The incubation at 37°C for 6 h was followed by trifluoroacetic acid addition to 0.1%. The whole solution was again subjected to preparative reversed-phase HPLC to isolate big ET-1.