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Secretion of Human Angiogenin into Periplasm and Culture Medium with Its Eukaryotic Signal Sequence by *Escherichia coli*

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Abstract : The synthesis and secretion of human angiogenin in *E. coli* by the natural leader sequence has been studied. We constructed a recombinant plasmid containing human angiogenin cDNA which encompassed all the coding region including leader sequence required for secretion. The recombinant plasmid was introduced into a suitable *E. coli* host. The angiogenin was detected in the culture medium and periplasm upon the induction of gene expression. The molecular weight of the secreted angiogenin was identical to that of authentic angiogenin purfied from human plasma when estimated by SDS-PAGE and immunoblotting, showing that the natural leader sequence was recognized and processed by the secretion machinery of *E. coli*. The angiogenin concentration in the culture medium reached a maximum within 2 h when expressed at 37°C with 0.02~2 mM IPTG. In contrast, the expression level increased gradually over time up to 11 h at 23°C with 0.002~2 mM IPTG and at 37°C with 0.002 mM IPTG.
Key words : eukaryotic leader sequence, *Escherichia coli*, human angiogenin, secretory expression.

The secretion of proteins is directed by N-terminal signal sequence which is removed by signal peptidase during secretion in both eukaryotic and prokaryotic cells. Most of the signal peptides are composed of three distinct regions: the amino-terminal positively charged region, central hydrophobic region, and carboxy-terminal cleavage region. In spite of the similarity between eukaryotic and prokaryotic secretion mechanism, few examples are reported about the secretion of eukaryotic protein in *E. coli* cells by natural leader sequence. It was shown that the human growth hormone was correctly processed and transported to the *E. coli* periplasm (Gray *et al.*, 1985). The expressed protein appeared to have correct disulfide bonds when analyzed by gel electrophoresis. The leader sequence of the immunoglobulin light chain was also reported to be compatible with the *E. coli* system (Zemel-Dreasen and Zamir, 1985). However, only a small fraction of the expressed protein was processed and transported into periplasm.

In this paper, we describe the synthesis, secretion and correct processing of human angiogenin in *E. coli* by its natural leader sequence. Angiogenin is a serum protein inducing the growth of a new blood vessel (Folkman and Klagsbrun, 1987). It has three disulfide bonds (Acharya *et al.*, 1994), and shows 35% amino acid sequence homology with bovine pancreatic ribonuclease (Strydom *et al.*, 1985). The precursor angiogenin is composed of 147 amino acids including 24 amino acids of signal sequence, and the mature protein does not contain any carbohydrate residue (Kurachi *et al.*, 1985). Expression of Met-(−1) human angiogenin was reported by Shapiro *et al.* (1988). A synthetic gene which encodes mature human angiogenin with N-terminal start codon was used for the cytoplasmic expression of angiogenin in *E. coli*. The protein was produced in an insoluble form, and active protein was obtained by denaturation and renaturation technique. It was also reported that authentic angiogenin was secreted into a culture medium of animal cell line transformed with the whole gene of human angiogenin (Kurachi *et al.*, 1988). The secretory expression of angiogenin protein in *E. coli* has not been reported yet. We constructed an expression vector encoding the natural human angiogenin precursor. The expressed angiogenin was processed, and secreted into the culture medium and periplasm of *E. coli*. The expression conditions are also discussed.

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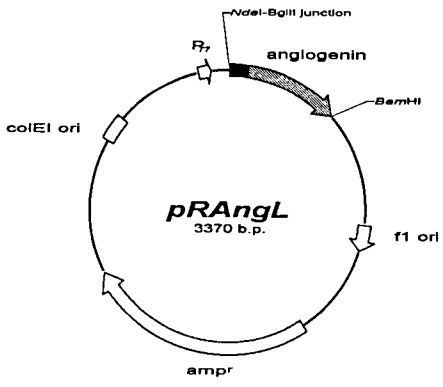

Fig. 1. Schematic illustration of expression vector pRAngL. The full length of angiogenin gene was placed on the downstream of T7 promoter. Also the ribosome-binding site exists just before the start codon. The filled box represents the natural leader sequence of angiogenin.

Materials and Methods

Construction of the expression vector

The cDNA of human angiogenin was amplified from the human liver cDNA library (Clontech, Palo Alto, USA) by PCR. The nucleotide sequences of the primers used for the PCR are 5'-GAA GAT CTA ATA TGG TGA TGG GCC TGG GC-3' for 5'-end and 5'-CGG GAT CCT TAC GGA CGA CGG AAA ATT GA-3' for 3'-end. The amplified DNA was digested with *Bgl*II and *Bam*HI, and a DNA fragment of ~0.4 kb was purified by electroelution from agarose gel. It was ligated with pRSET A (Invitrogen, San Diego, USA) vector digested with *Bgl*II, and the *E. coli* Top10 (Invitrogen, San Diego, USA) was transformed with the ligate. The recombinant vector containing angiogenin gene in a proper orientation was selected, and digested with *Nde*I and *Bgl*II to remove pRSET A leader sequence. The linear DNA was treated with S1 nuclease and self-ligated. The ligate was used to transform *E. coli* Top10. Finally, we obtained the desired vector pRAngL by screening transformants. Fig. 1 shows the schematic illustration of the constructed vector. The vector was introduced into *E. coli* BL21 (DE3) pLysS (Novagen, Madison, USA) for the expression of the cloned gene. All gene manipulation techniques followed the methods of Sambrook *et al.* (1989).

Expression and partial purification of angiogenin

The angiogenin gene was inserted downstream from the T7 promoter which promotes the expression of the inserted DNA with the action of T7 RNA polymerase provided by the host strain *E. coli* BL21 (DE3) pLysS. The cells were grown in LB medium (Sigma, St. Louis, USA) supplemented with 100 μg/ml of ampicilin, and the synthesis of T7 RNA polymerase was induced by IPTG (Promega, Madison, USA).

In order to determine the cellular location of expressed angiogenin, *E. coli* BL21 (DE3) pLysS containing pRAngL was grown at 37°C until OD_{600} reached ~0.8 and IPTG was added to a final concentration of 1 mM. The culture was continued for 4 h at 37°C, and the cells were harvested by centrifugation at 10000 $\times g$ for 15 min. The clear supernatant was concentrated with Centriprep-3 (Amicon, Beverly, USA). The periplasmic proteins were extracted by the method of Hsiung *et al.* (1986), and concentrated in the same way. After the extraction of periplasmic proteins, the cell pellet was resuspended in P buffer (10 ml P buffer for 100 ml culture), and lysed by the combined method of freezing-thawing and sonication to prepare cytosolic proteins. The cytosolic fraction was also concentrated with Cnetriprep-3. As a control, *E. coli* BL21 (DE3) pLysS was grown, and processed analogously.

The expressed angiogenin was partially purified by CM-52 (Whatman, Hillsboro, USA) column chromatography by the method of Fett *et al.* (1985) with some modifications. Briefly, the pH of the culture medium was brought to 6.6 with 1 N HCl, and the sample was applied to a CM-52 column equilibrated with P buffer (0.1 M sodium phosphate, pH 6.6). The column was washed with additional P buffer, and the adsorbed proteins were eluted with 1 M NaCl in P buffer. The 1 M NaCl elute was concentrated with Centriprep-3. The human angiogenin was purified from plasma by the same method described above.

Analysis of the expressed angiogenin

The concentration of angiogenin was estimated by the direct EIA method using anti-human angiogenin antibody (R&D systems, Minneapolis, USA). The immunoplates (Immulon 4 from Dynatech Laboratories, Chantilly, USA) were coated with samples containing angiogenin in PBSN buffer (0.1% sodium azide in phosphate-buffered saline). A blank well was prepared by applying PBSN instead of sample. The residual binding capacity was blocked with 0.1% bovine serum albumin (Sigma, St. Louis, USA) in washing buffer (0.05% Tween-20 in PBSN). Goat anti-angiogenin antibody was added at a concentration of 1 μg/ml in PBSN, and the immunoplates were incubated at room temperature for 1 h. Alkaline phosphatase-conjugated anti-goat IgG antibody (Sigma, St. Louis, USA, diluted 1:10000 with PBSN) was added, and the immunoplates were incubated at room temperature for 1 h. Absorbance at 405 nm was measured after 30 min of incubation with

p-nitrophenyl phosphate using a micoplate leader Emax (Molecular Devices, Menlo Park, USA). The immunoplates were washed three times with washing buffer following each step.

The expressed angiogenin was analyzed by SDS-PAGE using 15% gel. The specific band of angiogenin was detected by the immunoblotting technique. After elelctrophoresis, proteins were transferred to a nitrocellulose paper, and the paper was incubated in 0.1% BSA in washing buffer. It was treated with 1 μg/ml anti-angiogenin antibody and subsequently with alkaline phosphatase-conjugated anti-goat IgG antibody. The angiogenin band was visualized by incubating in 5-bromo-4-chloro-3-indolyl-1-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate.

The protein concentration was determined by the dye-binding method (Spector, 1978).

Results and Discussion

Cellular location of expressed angiogenin

The expression of angiogenin was induced by IPTG, and the cells were fractionated by the osmotic-shock method. The synthesized angiogenin was not detected in cytosol but in periplasm (Fig. 2), showing that the natural leader sequence directed the translocation of synthesized angiogenin. Moreover, some of the proteins were secreted into the culture medium at a concentration comparable to that of the periplasmic fraction (Fig. 2). Secretion of a protein into the medium by a Gram-negative bacterium requires translocation of the protein through the lipid bilayers of both the inner and outer membranes and the bacterial periplasm. The capacity of *E. coli* to secret proteins into the medium is limited to those entero-pathogenic strains (Pugsley, 1993). Generally, *E. coli* strains used for the expression of heterologous proteins are considered to be lacking in the terminal secretion machinery, and the bacteriocin or haemolysin system was used to secrete foreign proteins into extracellular space (Mackman *et al.*, 1987; Hsiung *et al.*, 1989).

In our experiment, however, the secretion of angiogenin into the medium was achieved without any specific secretion system. It was reported that proteins were often released into the medium probably by non-specific leakage from, or lysis of, the cell, and not by specific translocation through the outer membrane (Hockney, 1994). It is not clear whether angiogenin was secreted into the extracellular space by the action of its leader sequence or by any other non-specific leakage. One possible explanation comes from the fact that the *E. coli* strain used here contains the pLysS vector, which would express lysozyme by induction with IPTG. It can be assumed that the expression of lysozyme leads to the partial lysis of cells, resulting in the release of cellular proteins. However, several other chimeric

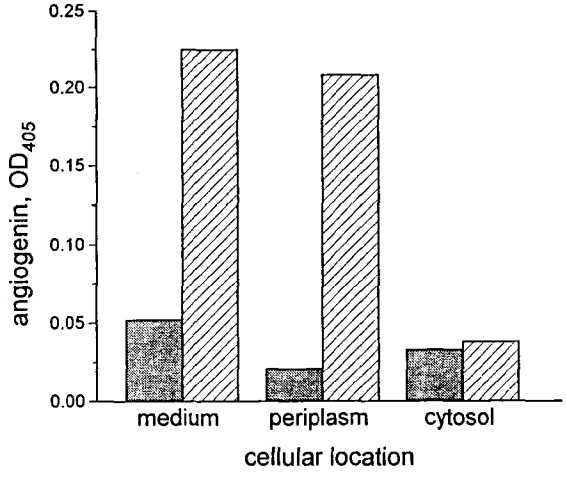

Fig. 2. Cellular location of angiogenin. *E. coli* BL21 (DE3) pLysS with (▨) or without (■) pRAngL was grown at 37°C in LB medium containing ampicilin until OD_{600} reached about 0.8, and expression of angiogenin was induced by 0.2 mM IPTG. The culture was processed as described in Materials and Methods to obtain medium, periplasmic, and cytosolic fractions, and analyzed by EIA to estimate angiogenin concentration. All the fractions were concentrated to a same volume for direct comparison.

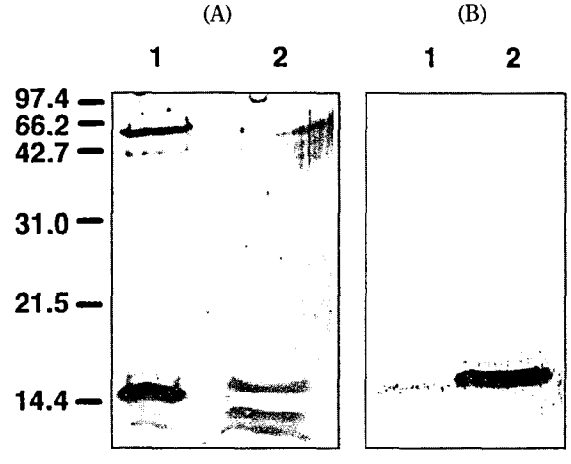

Fig. 3. SDS-PAGE and immunoblotting analysis of secreted angiogenin. (A) The culture medium containing secreted angiogenin was purified as described in Materials and Methods, and analyzed by SDS-PAGE with 15% gel. (B) The proteins were again analyzed by immunoblotting technique using anti-human angiogenin antibody. The lane 1 shows control angiogenin purified from human plasma, and lane 2 represents angiogenin obtained from *E. coli* culture medium. The size of the molecular weight maker proteins are denoted on the left side of the gel.

proteins containing the *E. coli* maltose binding protein were not released into the culture medium at all when the gene III signal sequence of bacteriophage M13 was used to translocate the secretion in the same *E. coli* strain (unpublished data). This shows that the secretion of angiogenin was not caused by cell lysis, but by a certain protein-specific mechanism.

Processing of angiogenin

The culture medium containing the secreted angiogenin was partially purified by CM-52 column chromatography, and the 1 M NaCl elute was analyzed by SDS-PAGE (Fig. 3a). A protein band was observed to have a similar migration rate as authentic angiogenin purified from human plasma. In order to identify the angiogenin band specifically, the same gel was alalyzed by immunoblotting using anti-human angiogenin antibody (Fig. 3b). The result clearly shows that the observed protein band is that of angiogenin. As shown here, the angiogenin obtained from *E. coli* frequently showed higher immunoreactivity than that purified from human plasma. This result can be partially explained by considering that the anti-human angiogenin antibody used here was raised with recombinant angiogenin obtained from *E. coli* by the method of Shapiro *et el.* (1988). The *E. coli*-derived recombinant human angiogenin is only partially blocked at its N-terminus by pyroglutamate, while the N-terminus of authentic human angiogenin is fully blocked (Strydom *et al.*, 1985; Shapiro *et al.* 1988). We also observed a significant difference between immunoreactivities of angiogenin preparations from different human plasma samples (data not shown). It is not clear whether this difference comes from a different purification condition or genetic variability of human angiogenin. The leader sequence of human angiogenin consists of 24 amino acids, and the relative molecular mass of the precursor form should be about 17,000, being much larger than that of mature angiogenin (M_r=14,000). If the leader sequence had remained intact, the secreted protein should have shown a significantly lower migration rate. Therefore, the leader sequence is thought to be recognized and processed by *E. coli* signal peptidase. However, the N-terminal amino acid sequence has to be determined in order to make it clear whether the cleavage site is identical to the human cell or not. The cleavage of leader sequence by *E. coli* signal peptidase requires a small residue at -1, a small or aliphatic residue at -3, and a helix breaker at -4 to -6 (Dalbey and von Heijne, 1992). The leader sequence of human angiogenin seems to satisfy these requirements, being Ala, Thr, Pro, and Pro respectively at the -1, -3, -4, and -5 position. After CM-52 chromatography, we obtained about 25 μg of angiogenin from 600 ml culture.

Expression condition

We tried to determine the optimal condition for extracellular secretion by varying expression conditions. When the proten was expressed at 37°C, the expression level reached a maximum within 2 h at IPTG concentrations higher than 0.02 mM (Fig. 4). Even at

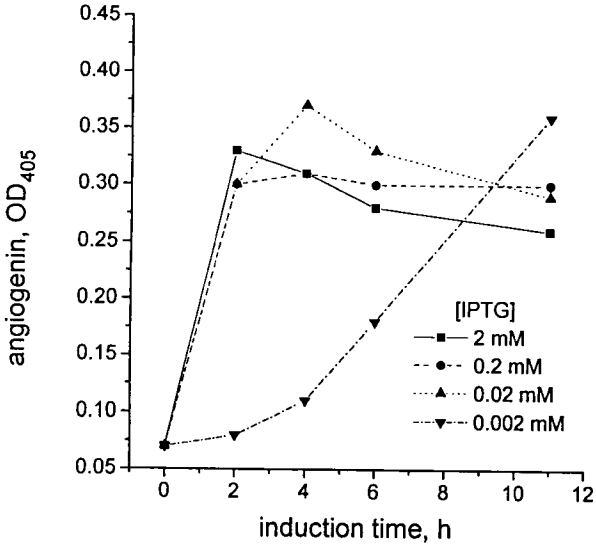

Fig. 4. Time course of expression at 37°C. The expression of angiogenin was induced at various IPTG concentration, and aliquotes were removed at time interval. The medium was harvested and analyzed for angiogenin concentration by EIA.

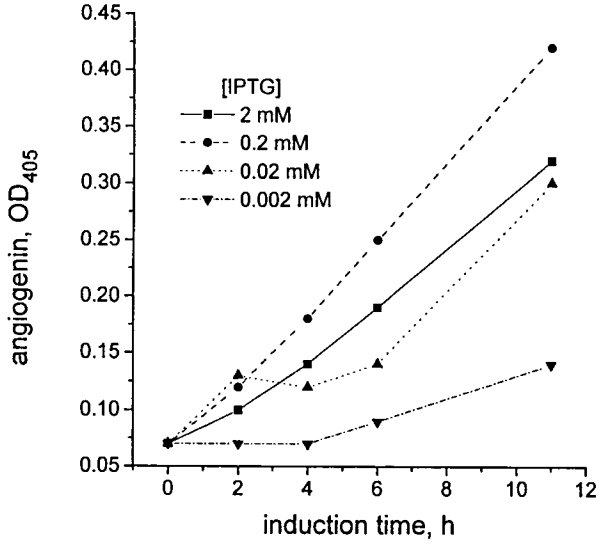

Fig. 5. Time course of expression at 23°C. The same experment was performed as described in Fig. 4 except that angiogenin was expressed at 23°C.

the maximum level, however, the angiogenin concentration in the culture medium was still low. By contrast, the concentration of angiogenin gradually increased at a lower IPTG concentration. Sometimes, a slow synthesis rate was proved to be benificial for the secretory expression of foreign proteins in *E. coli* (Takagi *et al.*, 1988). They achieved a higher level of secretory expression of subtilisin by lowering the induction temperature from 37°C to 23°C. Therefore, we tried to express angiogenin at 23°C, and the result is shown in Fig. 5. At all IPTG concentrations, the expression increased gradually over time. However, a longer expression time up to 24 h did not increase the level significantly (data not shown). And the optimal IPTG concentration for the secretory expression at 23°C was 0.2 mM.

Acknowledgements

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