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Expression, Purification, and Characterization of Prothrombin Kringle 2

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Previously, we reported that the prothrombin kringle 2 (fragment 2), induced by LPS administration into rabbit, inhibited bFGF-stimulated BCE cell growth (Lee *et al.*, 1998). In this study, we cloned and overexpressed the kringle 2 domain of rabbit and human prothrombin as a fusion protein with the *pel*B leader sequence in *E. coli* using the T7 promoter. The fusion protein was cleaved during translocation into the periplasmic space, and cleaved recombinant protein was readily isolated from whole cell lysate by DEAE-Sepharose and Sephacryl S-200 gel filtration chromatography. Both the recombinant rabbit and human prothrombin kringle 2 showed very similar biochemical and functional characteristics to the rabbit prothrombin kringle 2 purified from rabbit serum, in terms of abnormal electrophoretic migration and endothelial cell growth inhibitory activity.

Keywords: Angiogenesis inhibition, Purification, Recombinant prothrombin kringle 2,

Introduction

Kringles are small protein domains which have a characteristic three disulfide bonded secondary structure that was found in serine proteases which are involved in blood coagulation and fibrinolysis (Furie and Furie, 1988). The kringle domains have also been found in some other proteins which have biological functions different from the protease, like apolipoprotein (a) and hepatocyte growth factor (HGF) (Ikeo *et al.*, 1995).

DeSerrano *et al.* suggested that these kringle domains appear to be independent folding units, but the general functional role for kringles is not known (DeSarrano *et al.*, 1992). Because of the different properties of these apparently very similar polypeptides, recent efforts have been made to apply recombinant DNA technology to express kringle domains and to attempt to understand their structure-function relationships (DeSarrano *et al.*, 1992). Recently, Cao *et al.* reported that each domain of human plasminogen kringles showed anti-capillary endothelial cell proliferative activity which can be applied as an anti-cancer drug (Cao *et al.*, 1996; Boehm *et al.*, 1997). After their reports, many researchers became interested in the physiological function of kringle domains (Cao *et al.*, 1997).

To-date, 7 recombinant kringles have been successfully expressed in *E. coli* and their folding and ligand binding properties were examined. These include the kringle 1, 2, 3, 4, and 5 domains of human plasminogen, the kringle 4 domain of apoliporotein (a), and the kringle 1 domain of tissue type plasminogen activator (Marco *et al.*, 1982; Thewes, *et al.*, 1987; Cleary *et al.*, 1989; Menhart *et al.*, 1991; Li *et al.*, 1992; Marti *et al.*, 1994; Cao *et al.*, 1996; 1997). These works were assisted by the ability to purify in high yield the recombinant kringles from crude conditioned cell extracts on Sepharose-lysine affinity chromatography columns. Since it was believed that it is quite difficult to purify kringles, which do not specially adsorb to this type of column, an *E. coli* expression vector that would be useful for facile purification of such target molecule was constructed (DeSarrano *et al.*, 1992).

In this paper, we report that the rabbit and human prothrombin kringle 2 were properly expressed in *E. coli*, and that the expressed recombinant proteins were purified homogeneously by simple conventional column methods. The growth inhibitory activities of the recombinant human and rabbit prothrombin kringle 2 against bovine capillary endothelial cells are described.

Materials and Methods

Materials Restriction endonucleases were purchased from New

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England Biolab (Beverly, USA) and Promega (Madison, USA). Recombinant *Tca* polymerase was provided by Bioneer (Taejon Korea). Lysine-Sepharose 4B, DEAE-Sepharose fast flow, and Sephacryl S-200 were obtained from Pharmacia.

Bacteria strains and Plasmids *E. coli* XL1-blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46*thi relA*1 *lac*$^-$ F′ [*proAB*$^+$*lacI*^{q} *lacZ*ΔM15 Tn*10* (*tet*^{r})]) was used for routine transformations and plasmid preparations. *E. coli* strain BL21(DE3) pLysS (Novagen) was used for expression of kringle 2 under control of the T7 promoter. The plasmid pGEM-T for cloning of PCR product was purchased from Promega and expression plasmid pET22b+ was obtained from Novagen.

Plasmid manipulations Restriction enzymes were used according to the specifications supplied by the manufacturer. Plasmid preparations, agarose gel electrophoresis of DNA, ligation, and transformation of competent cells were as previously described (Lee *et al.*, 1998). Transformations were carried out by the procedure described by Hanahan (1983).

Induction of prothrombin kringle 2 gene expression in *E. coli* *E. coli* BL21(DE3) pLysS with the prET13 and phET13 (non-fusion) or pRE13 and pHE13 (fusion) which has the T7 promoter were incubated at a turbidity of 0.4 to 1.4 O.D. units at 600 nm in LB media, in the presence of 50 μg of ampicillin per ml, before being challenged with IPTG (isopropyl β-D-thiogalatopyranoside). IPTG was added to a final concentration of 0.1 to 5 mM and the cells were further incubated for 3 h. At the end of the incubation period bacteria were pelleted by centrifugation at 4000 $\times$ *g* for 15 min, and resuspended in a minimal volume of 10 mM sodium phosphate, pH 7.0, containing 50 mM NaCl and 1 mM phenylmethyl sulfonyl fluoride (PMSF). Cells were then disrupted by ultrasonication (Sonics Material Inc, Danburg, USA). The cell debris were removed by centrifugation at 13,500 $\times$ *g* for 10 min.

Purification of the recombinant prothrombin kringle 2 For the purification of the recombinant prothrombin kringle 2, the crude extract was chromatographed on DEAE-Sepharose column (2 $\times$ 12 cm) preequilibrated with a buffer containing 10 mM sodium phosphate, pH 7.0, 50 mM NaCl. With 150 mM (for rabbit kringle 2) or 180 mM (for human kringle 2) NaCl containing 10 mM sodium phosphate buffer, unbound and weakly bound proteins were removed. After the washing step, proteins were eluted with a 0–500 mM NaCl gradient in the above buffer at a rate of 20 ml/h. Fractions containing prothrombin kringle 2 were pooled and concentrated in the Centriprep device. The concentrated samples were loaded on a Sephacryl S-200 column (1.2 $\times$ 100 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and eluted at a rate of 18 ml/h. One unit of prothrombin kringle 2 was defined as the amount of protein, which decreases prothrombin turnover in 1 ml plasma at half maximal level. In brief, serially diluted prothrombin kringle 2 was mixed with the prothrombin free plasma. After 5-min incubation, various amounts of human prothrombin (Calbiochem La Jolla, USA) were added to the mixture. One unit of prothrombin was defined as the equal amount contained in one ml of human plasma. The activity of thrombin excised from the inactive precursor, prothrombin, was determined with the chromogenic substrate for thrombin, D-Phe-pipecolyl-Arg-p-nitranilide (Chromogenix S-2238), using a Uv-Vis spectrophotometer. Color development was determined by measuring the absorbance at 405 nm.

Amino acid sequence determination The amino acid sequence of the 12 N-terminal residues were determined by automated Edman degradation reactions in a protein sequencer (Applied Biosystems, Model 471A, USA).

Endothelial cell proliferation assay Endothelial cell proliferation assay was performed according to the method of O'Reilly *et al.* (Lee and Kim, 1998). Bovine capillary endothelial (BCE) cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% heat-inactivated bovine calf serum and 3 ng/ml recombinant human bFGF (basic fibroblast growth factor). Cells growing in gelatin-coated 6-well plates were dispersed in a 0.05% trypsin solution and resuspended with DMEM containing 10% bovine calf serum. Approximately 12,500 cells in 0.5 ml were added to each well of gelatinized 24-well plates and incubated at 37°C (in 10% CO_2) for 24 h. The media were replaced with 0.25 ml of fresh DMEM containing 5% bovine calf serum and samples were added to each well. After 30 min of incubation, media were added to obtain a final volume of 0.5 ml DMEM containing 5% bovine calf serum and bFGF at 1 ng/ml. After 72 h of incubation, the cells were trypsinized and counted using a hemocytometer.

Results

Construction of expression vectors For the recombinant study, plasmids for the large expression of recombinant prothrombin kringle 2 were generated by inserting appropriate cDNA under the control of T7 promoter in the pET22b+ vector (Fig. 1). When we induced recombinant kringle 2 expression from *E. coli* carrying prET13 and phET13 (non-fusion type), little or no expressed protein was detected (data not shown). Similar results were obtained when we used other expression hosts or vectors. It might be possible that the rabbit and human prothrombin kringle 2 mRNAs form a very strong secondary structure. The predicted secondary structure energies of rabbit and human prothrombin kringle 2 mRNA were -189.5 kcal/mol and -179.3 kcal/mol, respectively (DNAsis ver 7.0). In addition, *E. coli* usually produces very few proteins containing disulfide bonds, since the high intracellular concentrations of reduced glutathione favor disulfide reduction (DeLamarter *et al.*, 1985). Therefore, we decided to express kringle 2 by secretion into the periplasmic space where the redox potential for disulfide bond formation should be more favorable. By introducing 66 nt *pel*B leader sequence to the 5′ end of the prothrombin kringle 2 gene, we may reduce secondary structure within the mRNA and solve the problem of disulfide bond formation.

Optimization of prothrombin kringle 2 expression For maximizing the amount of recombinant kringle 2, we varied the culture conditions, such as temperature,

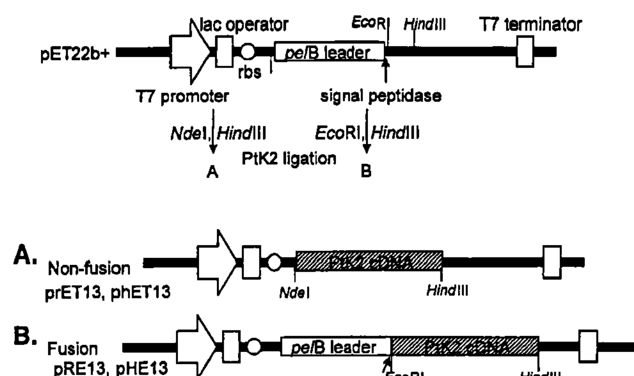


Fig. 1. Construction of the fusion type- (B) and non-fusion type- (A) expression vectors. Expression plasmid pET22b+ was obtained from Novagen.

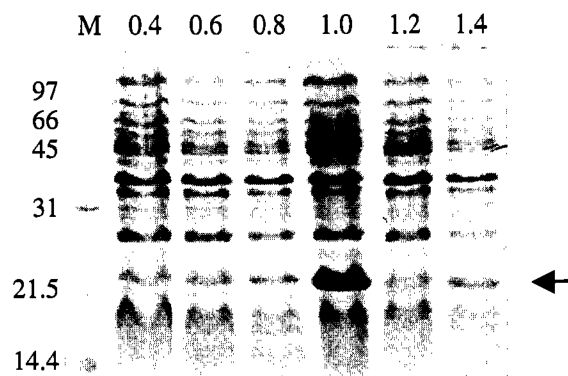


Fig. 2. Optimization of IPTG induction. Cultures of *E. coli* BL21 pLysS carrying expression plasmid pHE13 were grown in LB medium containing 50 μg ampicillin/ml. Lane M, Molecular weight standard (Bio-Rad). 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 indicate the crude extracts which were induced at the respective O.D. at 600 nm.

incubation time, and induction materials. The amount of expressed protein on SDS-PAGE was dependent on neither growth media nor temperature. However, the expression levels were quite varied on the cell density at the induction start point, e.g., the amount of induced prothrombin kringle 2 was maximum when IPTG (1mM) was added to the bacterial culture at A_{600} = 1.0. O.D. (Fig. 2) A successful induction was also achieved when we used 5 mM lactose as an inducer.

Another critical factor for optimization of expression was the incubation time after induction. The rate of synthesis of kringle 2 began to increase 0.5 to 2 h after induction and it remained at a high level for at least another 3 h (data not shown). However, total amount of protein in crude extracts gradually declined during this period. It might be caused by abnormal patterns of bacteria growth (Fig 3); 2.5 h after IPTG induction, bacteria showed a tendency to die. Similar trend was observed with 5 mM lactose induction (Fig 3). The extraordinary features of kringle 2 expression were also observed in human prothrombin kringle 2 expression.

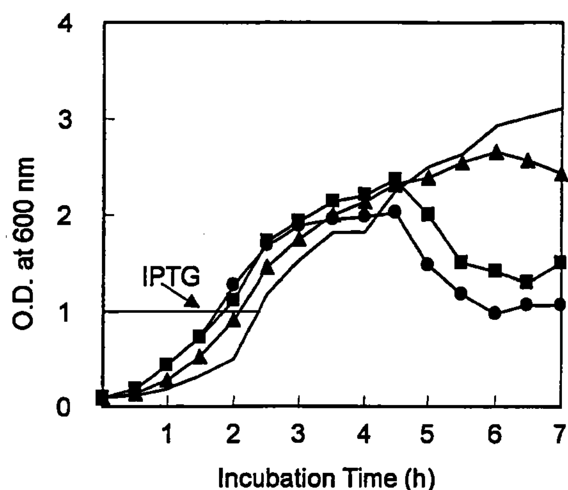


Fig. 3. Growth curve of *E. coli* expressing prothrombin kringle 2. Cultures of *E. coli* BL21(DE3) pLysis carrying expression plasmids pRE13 and pHE13 were grown in LB medium containing 50 μg ampicillin/ml. – control (not induced); ● and ■, *E. coli* carrying pRE13 and pHE13 induced with IPTG, respectively; ▲, *E. coli* carrying pRE13 induced with 5 mM lactose.

Purification of recombinant prothrombin kringle 2 Rabbit prothrombin kringle 2 was absorbed on a DEAE-Sepharose column and eluted with a step gradient of sodium chloride (Fig. 4A). Human prothrombin kringle 2 also bound to DEAE-Sepharose column. Since the calculated pI value of human prothrombin kringle 2 (pI=4.03) is lower than rabbit kringle 2 (pI=4.52), the human prothrombin kringle 2 needs a higher concentration of sodium chloride (Fig. 4B).

Active fractions were pooled and concentrated by a centriprep device. The concentrated sample was loaded on Sephacryl S-200 column and the rabbit and human prothrombin kringle 2 were eluted at fraction numbers 30 to 35 (Fig. 5). These elution profiles were well matched with those of the prothrombin kringle 2 purified from rabbit serum (Lee *et al.*, 1998).

In order to confirm that the cDNA sequence of rabbit and human prothrombin kringle 2 were faithfully translated, the amino acid sequence analysis was performed with the purified recombinant prothrombin kringle 2. From this sequence analysis, we found the fusion proteins were cleaved during translocation into the periplasmic space, and the result of the N-terminal amino acid sequence analysis agreed well with the deduced amino acid sequence from the cDNA sequence of rabbit and human prothrombin kringle 2 (Table 1) (Lee *et al.*, 1998).

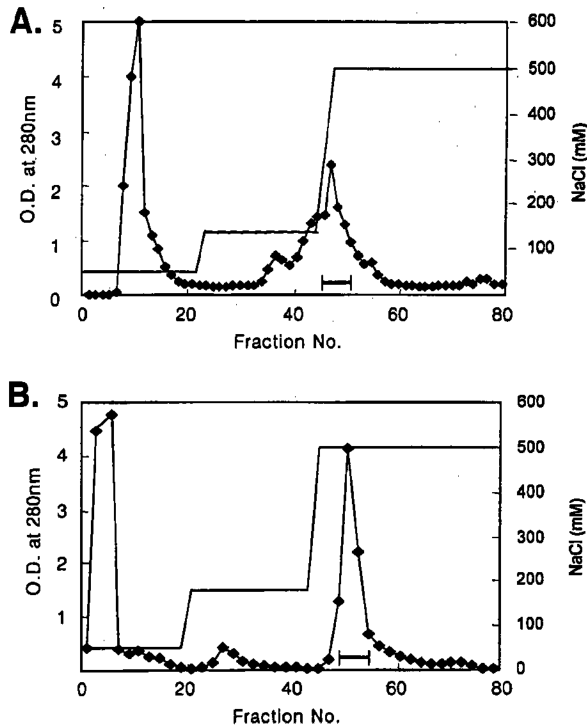


Fig. 4. Purification of recombinant prothrombin kringle 2 from *E. coli*. A. DEAE-Sepharose ion-exchange chromatography of recombinant rabbit kringle 2. The column was equilibrated and washed with 10 mM sodium phosphate (pH 7.0) containing 50 mM NaCl. B. DEAE-Sepharose ion-exchange chromatography of recombinant human kringle 2. Human kringle 2 was eluted at 500 mM NaCl step gradient. (—), the kringle 2-containing fractions.

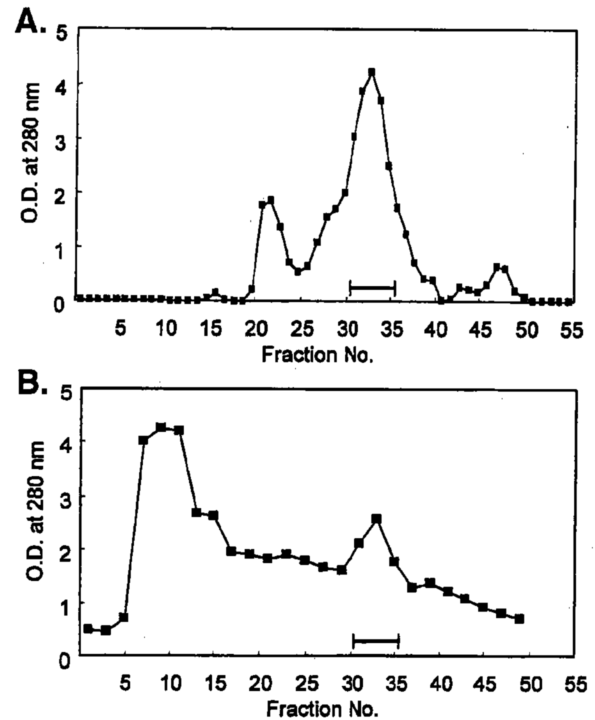


Fig. 5. Purification of recombinant prothrombin kringle 2 from *E. coli*. A. Sephacryl S-200 gel filtration chromatography of recombinant rabbit prothrombin kringle 2. B. Sephacryl S-200 gel filtration chromatography of recombinant human prothrombin kringle 2. (—), the kringle 2-containing fractions.

Table 1. cDNA sequences and N-terminal amino acid sequences of recombinant prothrombin kringle 2.

	Sequence
Human	*MDIGINSDPN* S E G S S V N L S P P L E 5′ TCCGAAGGCTCCAGTGTGAATCTGTCACCTCCATTGGAG
Rabbit	*MDIGINSDPNS* T G H S G V T Q P P P L E 5′ ACTGGACACTCCGGGGTGACTCAGCCCCCGCCACTGGAG

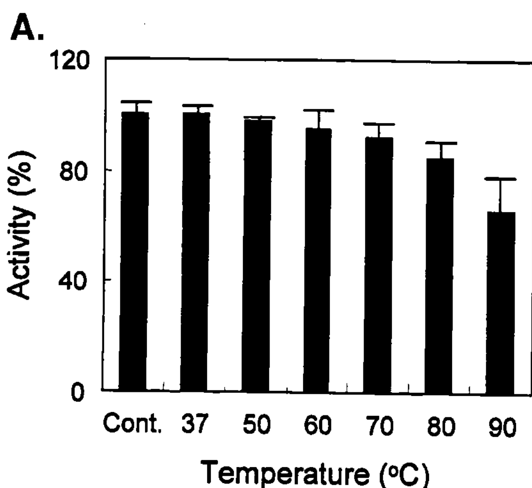
Underlined amino acid sequences came from multiple cloning sites.

Characteristics of recombinant prothrombin kringle 2 As mentioned in the introduction, only 7 recombinant kringles have been successfully expressed in *E. coli*, to date. All of them were purified by assistance of lysine-Sepharose column chromatography (DeSarrano *et al.*, 1992). It is believed that prothrombin kringles do not bind to the ω-amino group of lysine, since the prothrombin do not absorb to columns of Sepharose-lysine. In fact, purified recombinant prothrombin kringle 2 did not bind to a lysine-Sepharose column (data not shown).

Meanwhile, the thermal stability of the recombinant prothrombin kringle 2 has been examined through the prothrombin activation inhibition assay at various temperatures. The recombinant prothrombin kringle 2 appears to be extremely heat stable below 70°C (Fig. 6A). When the conditioned crude extract of prothrombin kringle 2 was applied to heat, other protein bands disappeared, but recombinant prothrombin kringle 2 remained in the soluble fraction (Fig. 6B). Making a good use of heat stability of the protein, recombinant prothrombin kringle 2 could be easily purified only by Sephacryl S-200 gel filtration (data not shown).

Abnormal mobility on SDS-PAGE The purified prothrombin kringle 2 migrated as a single band on SDS-PAGE under reducing conditions (Fig. 7, lane 1). The purified protein was judged to be better than 95% homogeneous after densitometer-scanning of the gel stained with Coomassie brilliant blue G.

The most interesting thing is that the reduced rabbit prothrombin kringle 2 migrated to a two-fold higher position with a molecular mass of 22 kDa compared with



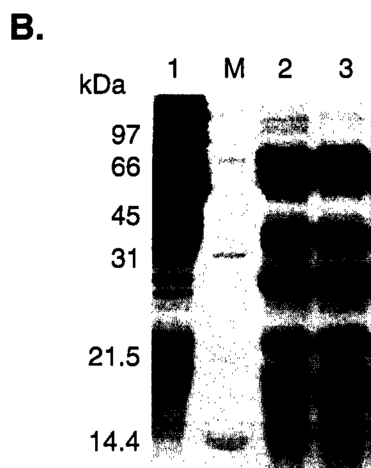


Fig. 6. Change of the prothrombinase complex inhibitory activity with heat treatment. A. The purified prothrombin kringle 2 was incubated 10 min at various temperatures (37, 50, 60, 70, 80, 90°C). B. Electrophoregram of heat-treated crude extracts. M, molecular weight standard; lane 1, control crude extract; lane 2, 50°C for 10 min; lane 3, 60°C for 10 min.

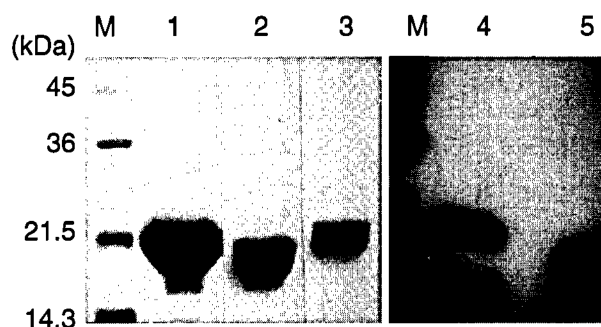


Fig. 7. SDS-PAGE analysis of the purified recombinant prothrombin kringle 2. Lane M, molecular weight standard; lane 1, reduced recombinant rabbit prothrombin kringle 2; lane 2, non-reduced recombinant rabbit prothrombin kringle 2; lane 3, 8 M urea gel electrophoresis; lane 4, reduced recombinant human prothrombin kringle 2; lane 5, non-reduced recombinant human prothrombin kringle 2.

its molecular mass calculated from its amino acid sequence. As shown in Fig 7, the reduced rabbit and human prothrombin kringle 2 migrated to a higher position, with a molecular mass of about 22 kDa and 21 kDa (lanes 1, 4), compared with the non-reduced prothrombin kringle 2 with a molecular mass of 18 kDa and 17 kDa (lanes 2, 5). However, urea did not affect on the protein mobility on polyacrylamide gel electrophoresis (lane 3). Considering their molecular masses calculated from their amino acid sequences (13.2 kDa and 13.8 kDa, respectively), this phenomena is quite abnormal. This has been observed in the prothrombin kringle 2 which was purified from rabbit serum (Lee *et al.*, 1998) and in the first kringle domain of angiostatin which suppresses endothelial cell growth more potently than the other kringle domains of angiostatin (Cao *et al.*, 1996). The abnormal migration of these molecules on SDS-PAGE under reducing conditions may be caused by some unusual inherent property of the protein, such as the presence of a high number of proline residues in the molecules (Pham and Sivasubramanian, 1992). In addition, a high number of glutamate residues on the C-terminal portion of the molecule may also be responsible (Kaufmann *et al.*, 1984).

Dose dependent-specific inhibition of capillary endothelial cells Purified rabbit and human prothrombin kringle 2 were assayed for their activities on BCE cell growth stimulated by bFGF. As shown in Fig. 8, recombinant rabbit prothrombin kringle 2 inhibited BCE cell proliferation in a dose-dependent manner. The concentration of recombinant rabbit prothrombin kringle 2 required to reach 50% inhibition (ED_{50}) was about 150 nM. The recombinant human prothrombin kringle 2 exhibited higher endothelial cell growth inhibitory activity (approximately 120 nM) than the recombinant rabbit prothrombin kringle 2 and purified rabbit prothrombin kringle 2 against BCE cells (Table 1).

Discussion

Recently, we purified a protein molecule which showed anti-endothelial cell proliferative activity from the LPS-stimulated serum of New Zealand white rabbits. The purified protein was identified as a prothrombin kringle 2 domain (or fragment-2) using Edman degradation and the amino acid sequence deduced from the cloned cDNA (Lee *et al*, 1998b). In this paper, we show how the recombinant rabbit and human prothrombin kringle 2 were prepared, which showed very similar biochemical characteristics to rabbit kringle 2 purified from rabbit serum. Also, we report that not only recombinant rabbit kringle 2 but also

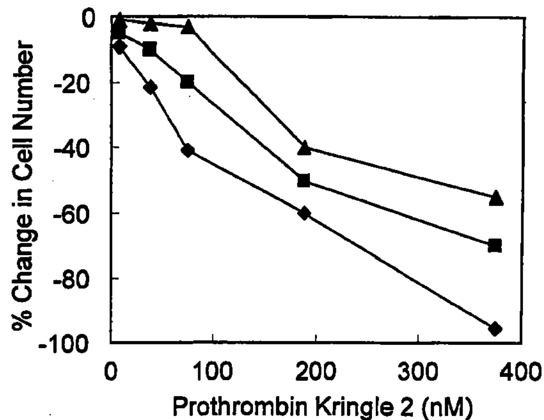


Fig. 8. Comparison of endothelial cell growth inhibitory activity of recombinant rabbit and human kringle 2 with prothrombin kringle 2 purified from rabbit sera (Lee *et al.*, 1998). Recombinant rabbit prothrombin kringle 2 (■), purified rabbit kringle 2 (▲), and recombinant human prothrombin kringle 2 (◆) were assayed on BCE cells in the presence of 1 ng/ml bFGF in a 72-h proliferation experiment. Values represent the means of three determinations (± SE) as a percentage of inhibition.

Table 2. The half-maximal concentration (ED_{50}) of prothrombin kringle 2 and other known endothelial cell growth inhibitory molecules.

Protein	ED_{50} (nM)
Recombinant rabbit prothrombin kringle 2	150
Recombinant human prothrombin kringle 2	120
Rabbit prothrombin kringle 2 (purified)[1]	155
Angiostatin[2]	140
Angiostatin kringle 1	320
Angiostatin kringle 3	460

[1] Lee *et al.*, 1998
[2] Cao *et al.*, 1996

recombinant human kringle 2 showed anti-endothelial cell growth activity.

From the above data, we may say that recombinant rabbit and human prothrombin kringle 2 folded properly in bacterial periplasmic space. The recombinant protein showed the same or higher cell growth inhibitory activity as the native prothrombin kringle 2 which was purified from rabbit serum. Therefore, these expression and purification systems are quite useful for studying structure and function relationships of prothrombin kringle 2. The recombinant prothrombin kringle 2 showed very similar physical properties. In particular, abnormal mobilities on SDS-PAGE were observed both in rabbit and human kringle 2.

O'Reilly *et al.* (1994) reported that the first four kringle domains of human plasminogen, named angiostatin, are a potent inhibitor of angiogenesis *in vitro* and *in vivo*. Considering the fact that the acquired drug resistance is a major problem in anti-cancer therapy, the anti-angiogenic therapy by endogenous kringle molecules could attract much attention (Boehm *et al.*, 1997). In this regard, rabbit and human prothrombin kringle 2 have a possibility to become a novel candidate for cancer therapy, since the ED_{50} values of rabbit and human prothrombin kringle 2 were more or less the same as those of angiostatin and its kringle domain (Table 2).

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