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Secretory Production of Recombinant Urokinase Kringle Domain in *Pichia pastoris*

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Abstract Human urokinase kringle domain, sharing homology with angiostatin kringles, has been shown to be an inhibitor of angiogenesis, which can be used for the treatment of cancer, rheumatoid arthritis, psoriasis, and retinopathy. Here, the expression of the kringle domain of urokinase (UK1) as a secreted protein in high levels is reported. UK1 was expressed in the methylotrophic yeast *Pichia pastoris* GS115 by fusion of the cDNA spanning from Ser47 to Lys135 to the secretion signal sequence of α-factor prepro-peptide. In a flask culture, the secreted UK1 reached about 1 g/l level after 120 h of methanol induction and was purified to homogeneity by ion-exchange chromatography. Amino-terminal sequencing of the purified UK1 revealed that it was cleaved at the Ste13 signal cleavage site. The molecular mass of UK1 was determined to be 10,297.01 Da. It was also confirmed that the purified UK1 inhibited endothelial cell proliferation stimulated by basic fibroblast growth factor, vascular endothelial growth factor, or epidermal growth factor, in a dose-dependent manner. These results suggest that a *P. pastoris* system can be employed to obtain large amounts of soluble and active UK1.

Key words: Angiogenesis, urokinase, kringle, inhibitor, *Pichia pastoris*

Numerous studies have shown that both primary tumor and metastatic growth depend on angiogenesis [6, 8]. Angiogenesis usually occurs during development but, in an adult, it is involved in tissue regeneration and chronic inflammatory conditions including cancer. Targeting endothelial cells that support tumor growth, rather than cancer cells themselves, is a promising therapeutic strategy to overcome drug resistance induced by cytotoxic chemotherapeutic drugs [7]. Various angiogenesis inhibitors have been developed to block tumor angiogenesis [13, 19, 20]. Angiostatin, bevacisumab, canstatin, combrestastin, endosatin, NM-3, 2-methoxyestradiol, and vitaxin are in clinical trials as direct-acting angiogenesis inhibitors [13]. Angiogenesis inhibitors can also be used for the treatment of rheumatoid arthritis, psoriasis, and retinopathy [6, 9, 15, 24].

Among these inhibitors, angiostatin is one of the most potent angiogenesis inhibitors, and was first isolated from tumor-bearing animals [23]. *In vitro*, it specifically inhibits endothelial cell proliferation, but not proliferation of other cell types, including tumor cells [4, 23]. *In vivo*, angiostatin suppresses neovascularization and tumor growth in animals without displaying toxic effects [18, 22, 23]. It consists of the first four kringle domains of plasminogen and can be generated by limited proteolysis [18, 23]. Kringle domains are protein modules composed of 78–80 amino acids connected by a characteristic triple disulfide-linked loop. The triple disulfide bonds are strictly conserved between kringles [5].

The kringle domain of urokinase (UK1), which shares about 30–40% amino acid sequence homology with angiostatin, has been also shown to elicit inhibitory activity on angiogenesis [16]. In the previous work, the UK1 protein was expressed by a bacterial expression and refolding process. Although these processes can produce folded protein, the insolubility of prokaryotic recombinant proteins and the requirement of the refolding process often decrease the yield of soluble and active proteins. Endotoxin removal also should be cautiously considered. To overcome these problems, expression of UK1 in *Pichia pastoris* was performed. Here, the secretory production of the recombinant human UK1 in *P. pastoris* and a simple procedure for its

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purification is described, and its inhibitory activity on endothelial cells is also confirmed.

METERIALS AND METHODS

Materials

Restriction enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany), and ligase from Takara (Shiga, Japan). The reagents used for cell culture, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin solution were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) were purchased from R&D systems (Minneapolis, MN, U.S.A.).

Strains and Plasmids

E. coli TOP10F', pPICZα-C vector, and *P. pastoris* GS115 were purchased from Invitrogen (EasySelect™ *Pichia* Expression Kit). *E. coli* TOP10F' was used as a host strain for constructing pPICZα-C/UK1. pPICZα-C contains the alcohol oxidase1 (AOX1) promoter, zeocin selectable marker, and α-factor signal sequence derived from *S. cerevisiae*. *P. pastoris* GS115 was selected as a host strain for expression.

Media

E. coli TOP10F' was grown in low salt LB (1% tryptone, 0.5% NaCl, 0.5% yeast extract, pH 7.0). BMGH (buffered minimal medium containing glycerol and histidine; 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 0.4% histidine) and BMMH (buffered minimal medium containing methanol and histidine; 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, 0.4% histidine) were used for growing *P. pastoris* and producing the recombinant UK1. MDH agar (minimal dextrose medium containing glucose and histidine; 1.34% YNB, 4×10^{-5}% biotin, 2% dextrose, 0.4% histidine, 1.5% agar) and MMH agar (minimal mediumn containing methanol and histidine; 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, 0.4% histidine, 1.5% agar) plates were used for Mut (Methanol Utilization) screening.

Construction of an Expression Vector

A 267 bp DNA fragment encoding the amino acids spanning from Ser^{47} to Lys^{135} of human urokinase was amplified by polymerase chain reaction (PCR) with Pfu polymerase (Stratagene, La Jolla, CA, U.S.A.) and two primers; forward primer A (5'-CAGTATCGATCTCAAAAACCT-GCTATGAG-3') and reverse primer B (5'-CTGATCT-AGATCATTTTCCATCTGCGCAGTC-3'). The PCR product was digested with *Cla*I and *Xba*I and ligated to pPICZα-C linearized with the same enzyme set and dephosphorylated. The ligated DNA was used to transform *E. coli* TOP10F' (Fig. 1). The sequence of the insert DNA was confirmed by DNA sequencing. The resulting recombinant plasmid, pPICZα-C/UK1, has additional amino acids at the amino-terminus, if it is cleaved at the Ste13 signal cleavage site: Ser and Ile.

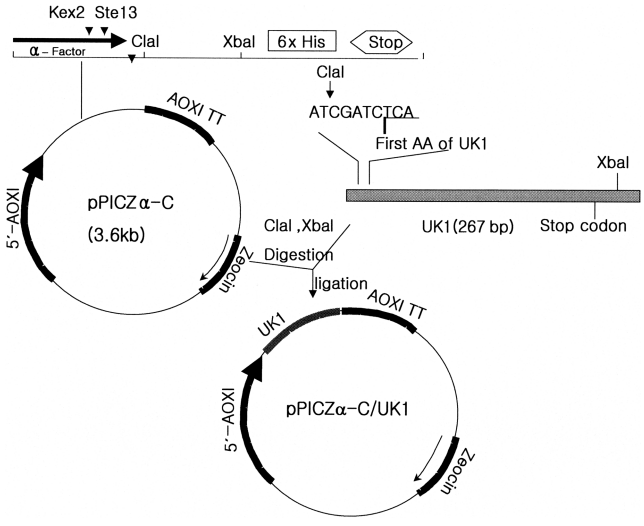

Fig. 1. Construction of a UK1 expression plasmid.

Transformation of *P. pastoris*

P. pastoris GS115 was transformed with pPICZα-C/UK1 linearized with *Sac*I. Approximately 3 μg of the linearized plasmid DNA was used for electroporation in 0.2 cm cuvettes at 1.5 kV, 25 μF, 400 Ω, using a Gene Pluser (Bio-Rad, Hercules, CA, U.S.A.). Immediately after pulsing, 1 ml of cold 1 M sorbitol was added to the cuvette, and the mixture was incubated at 30°C for 1 h without shaking. Cells were plated onto YPD (yeast extract peptone dextrose medium, 1% yeast extract, 2% peptone, 2% dextrose) agar containing 1,000 μg/ml zeocin. Resultant clones were screened for expression.

Determining the Mut Phenotype

To screen for methanol utilization, transformants were streaked on MMH and MDH agar plates. Mut^+ control (GS115/HIS+/β-galactosidase, Invitrogen) and Mut^s control (GS115/His^+/albumin, Invitrogen) were included for comparison. After incubation at 30°C for 48 h, Mut^+ colonies were visibly larger than the Mut^s transformants.

Screening for UK1 Expression

P. pastoris transformants were cultured in 10 ml of the BMGH at 30°C in a shaking incubator (250 rpm) until the culture reached OD_{600}=3. Cells were collected by centrifugation and resuspended in 2 ml of BMMH. Methanol was added every 24 h to a final concentration of 0.5%. The secretion of UK1 was screened by SDS-PAGE of the culture broth and Coomassie blue staining.

Expression and Purification of UK1

The expression of UK1 protein in large scale was carried out in a 2-liter shaker flask. *P. pastoris* [pPICZα-C/UK1] was cultured in 500 ml of the BMGH at 30°C in a shaking incubator (250 rpm) until the culture reached OD_{600}=3. Cells were collected by centrifugation at 1,500 ×g for 5 min, and resuspended in 100 ml of BMMH. The pure methanol was added every 24 h to a final concentration of 0.5%. After 120 h, cells were centrifuged at 1,500 ×g for 5 min, and culture broth was stored at −70°C until use. Crude culture broth (200 ml) containing UK1 protein was clarified by centrifugation at 14,000 ×g for 20 min, concentrated, and the buffer was changed with 20 mM phosphate buffer (pH 7.0) containing 50 mM NaCl by ultrafiltration using YM3 membrane (Millipore, Billerica, MA, U.S.A.). The sample was dialyzed against 20 mM phosphate buffer (pH 7.0) containing 50 mM NaCl and applied onto the Vivapure S spin column (Vivascience AG, Hannover, Germany) equilibrated with the same buffer. After centrifugation at 500 ×g for 5 min, the column was washed with the same buffer, and UK1 protein was eluted with 20 mM phosphate buffer (pH 7.0) containing 1 M NaCl. The eluted sample was intensively dialyzed against deionized water and lyophilized.

Polyclonal Antibody Generation and Western Blot Analysis

A polyclonal antiserum against human urokinase kringle domain was raised by injecting BALB/c mice three times with 100 μg of the recombinant UK1 protein derived from bacterial expression and refolding [16]. IgG was purified from this antiserum by protein-A chromatography and stored at 1 mg/ml. Protein samples were resolved in 14% SDS-PAGE and electroblotted onto nitrocellulose membranes. Polyclonal antibody raised against the recombinant UK1 was used in the primary binding reaction. Primary antibody/antigen complexes were detected using goat anti-mouse IgG antiserum conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), and developed using West-Zol™ chemiluminescence reagent (iNtron Biotechnology, Kyungki-Do, Korea).

Analysis of Oxidation State

To determine the presence of putative disulfide bonds, the purified recombinant UK1 protein was treated with β-mercaptoethanol-containing buffer. A control sample was prepared without β-mercaptoethanol. Both protein samples were analyzed by SDS-PAGE.

Analysis of Protein

Amino acid sequence was determined with an Applied Biosystems model, Procise 491 protein sequencing system at the Korea Basic Science Institute (Seoul, Korea). Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was performed using a Voyager Biospectrometry work station with a linear mass analyzer (PerSeptive Biosystems, Framinghum, MA, U.S.A.) at the Korea Basic Science Institute [14].

Cell Proliferation Assay

Human umbilical vein endothelial (HUVE) cells were isolated from fresh cords by an adaptation of the method described by Jaffe *et al.* [10], and maintained in M199 medium containing 20% FBS, 30 mg/ml endothelial cell growth supplements (Sigma, St. Louis, MO, U.S.A.), 25 mM Hepes, 2.2 g/l sodium bicarbonate, 2 mM L-glutamine, and 1% antibiotics. Cells at passage 3, 4, or 5 were used for the experiments.

Cells at 20,000 cells/well were plated into gelatinized 24-well culture plates and incubated in M199, 10% FBS, 90 μg/ml heparin, and 1% antibiotics for 24 h. The medium was replaced with 0.25 ml of M199, 5% FBS, 90 μg/ml heparin, and 1% antibiotics, and the test sample was applied. After 30 min of incubation, 0.25 ml of M199, 5% FBS, 90 μg/ml heparin, 1% antibiotics, and a growth factor (6 ng/ml bFGF, 20 ng/ml VEGF, or 20 ng/ml EGF) was added to each well. After 18 h, 1 μCi (0.037 MBq) of [^{3}H]-thymidine was added to each well. After further incubation for 6 h, cells were fixed with methanol, washed three times with cold 10% trichloroacetic acid solution, and dissolved in 0.25 N NaOH and 1% SDS. Radioactivity was determined using a liquid scintillation counter. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Expression and Purification of UK1

Employing *P. pastoris*, a methanotrophic strain, has some advantages, including facilitated secretion of the expressed protein in the medium and low level secretion of endogenous host protein in the yeast strain (GS115). In order to increase production of soluble and active recombinant protein and simplify the purification process, UK1 was expressed in *Pichia pastoris*. The recombinant plasmid constructed for the UK1 expression was designed to span from Ser47 to Lys135 of the human urokinase molecule without any artificial tag-sequences (Fig. 1). The pPICZα-C vector was selected for expression. The cDNA was replaced in-frame with the amino acids of α-factor signal sequence, producing a fusion between it and the sequence coding for the α-factor secretion signal peptide. Heterologous proteins fused to the sequence are cleaved at Arg and Glu in the sequence Glu-Lys-Arg-Glu-Ala-Glu-Ala by the KEX2 endopeptidase which cleaves on the carboxyl side of dibasic residues [11]. The Glu-Ala spacer repeats at the N-terminus are then removed by the STE13 dipeptidyl amino peptidase [1, 3, 12].

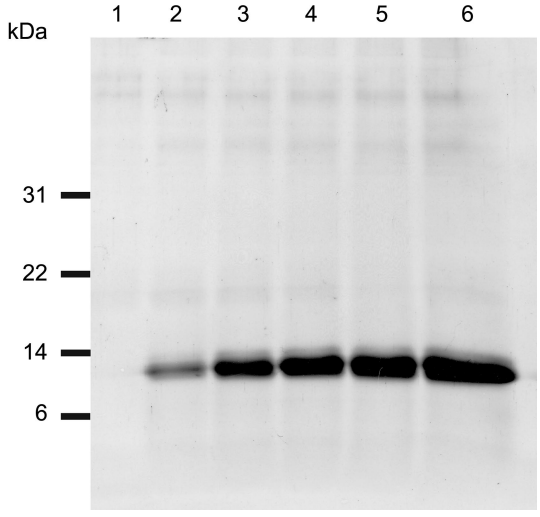

Fig. 2. Time-course for UK1 secretion in shake-flask cultures of a *Pichia* transformant.
Cultures were induced in YNB-methanol pH 6.0+casamino acids. Ten ml of each culture broth was subjected to SDS-PAGE analysis, after 0 h (lane 1), 24 h (lane 2), 48 h (lane 3), 72 h (lane 4), 96 h (lane 5), and 120 h (lane 6).

For the selected zeocin-resistant colonies, initial screening by SDS-PAGE analysis was carried out to identify yeast clones with high levels of expression. Since all of the highly-producing strains picked were identified as Mut^S strains, the Mut^S strain was chosen for the recombinant protein production. In a flask culture, UK1 was initially detected 24 h after the start of methanol induction (Fig. 2), and its level was continuously increased over a period of 120 h. The expression level was assessed as ~1 g/l after 120 h of methanol induction. The produced UK1 was then purified to apparent homogeneity in a single step by S spin column chromatography (Fig. 3).

In the previous work, initial levels of UK1 expression in *E. coli* were not high, and the protein required solubilization before refolding. Typical final yields were about 10 mg/l which was a relatively low level [16]. However, from the present study of producing UK1 in *P. pastoris*, UK1 could be obtained by a simple purification procedure with a high final yield (about 350 mg/l). Expression of a single kringle domain, such as the recombinant kringle 2 domain of tissue-type plasminogen activator in *Pichia*, has also been shown to provide the protein secreted at high levels, allowing easy purification of several hundred mg from shake flasks [21]. In addition, angiostatin consisting of kringles 1–4 of plasminogen was successfully produced by large-scale fermentation of *Pichia* transformants (140 g/2000-l scale) [25]. Taken together, these results indicate that the *Pichia pastoris* expression system is efficient for expression of kringle domains.

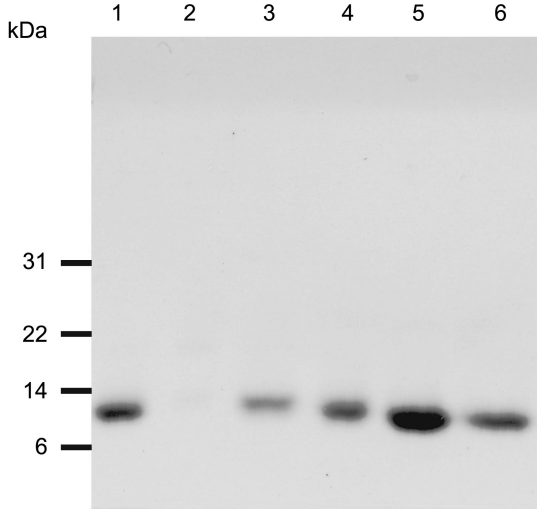

Fig. 3. Purification of the recombinant UK1 by S spin column chromatography.
Lane 1, crude UK1 fraction; lane 2, flow-through fraction; lanes 3 & 4, fractions washed with 20 mM potassium phosphate buffer (pH 7.0) containing 50 mM NaCl; and lanes 5 & 6, fractions eluted with 20 mM potassium phosphate buffer (pH 7.0) containing 1 M NaCl.

Characterization of the Purified UK1

To characterize the purified UK1, SDS-PAGE analysis was performed under reducing and nonreducing conditions. UK1 contains six cysteine residues that form three disulfide bonds [5]. The purified UK1 migrated as a single band of about 11–12 kDa under reducing condition (Fig. 4A), and it migrated a little faster due to the oxidation state of disulfide bonds under nonreducing condition.

Expression of the recombinant UK1 protein was confirmed by Western blot analysis using the polyclonal antibody raised against the recombinant UK1 which was obtained

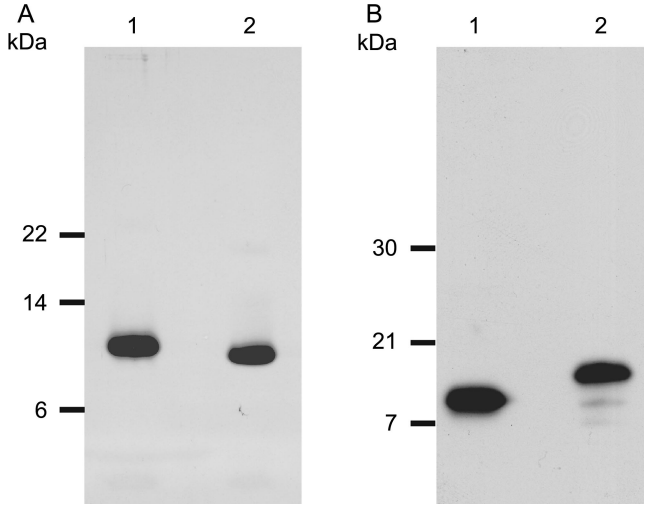

Fig. 4. Electrophoretic analysis of the redox state of the recombinant UK1 protein and immunoreactivity analysis.
(A) The purified proteins were analyzed under reducing (lane 1) and nonreducing (lane 2) conditions. (B) Western blot analysis of the recombinant UK1 proteins expressed from *P. pastoris* (lane 1) and *E. coli* (lane 2).

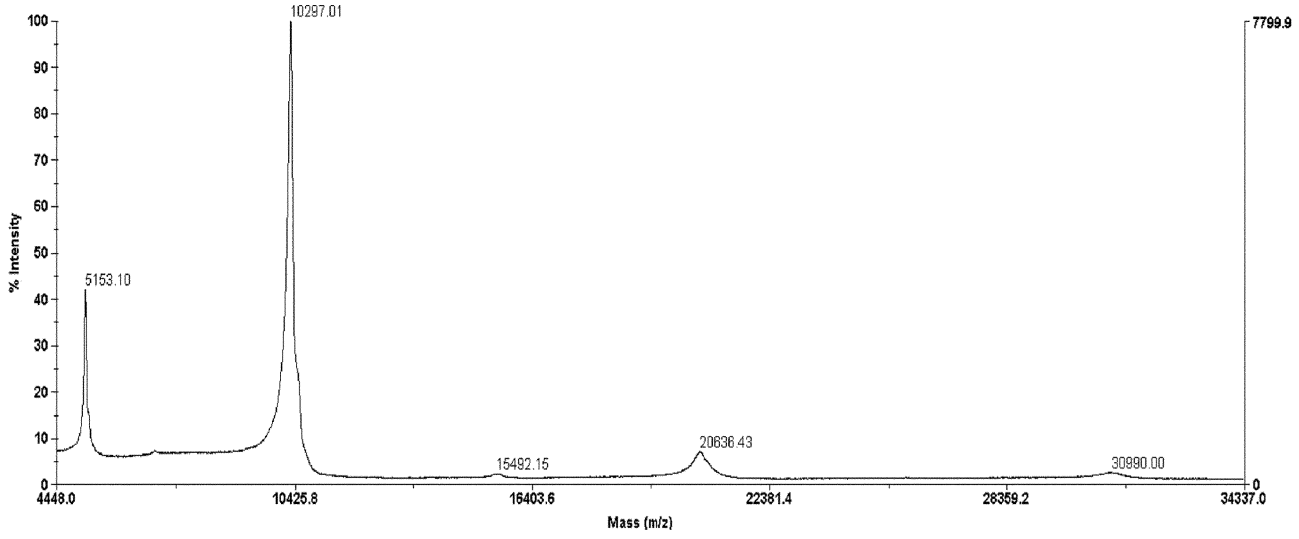

Fig. 5. Mass spectrum of the purified UK1.
The MALDI mass spectrum is dominated by a single component (second peak) with a measured molecular mass of 10,297.01 Da.

from a prokaryotic expression system [16] (Fig. 4B). Since the UK1 protein produced in a bacterial system carries an artificial His-tag sequence at the carboxyl terminus and four additional amino acid residues, its calculated molecular mass of 11,595 Da is larger than that of the UK1 protein (10,348.7 Da) produced in *Pichia*. Consequently, as seen in Fig. 4B the apparent difference of molecular mass between the recombinant UK1 proteins produced by yeast and bacteria was detected.

Amino-terminal sequencing of the secreted UK1 showed that the exact N-terminal sequence was Ser-Ile-Ser-Lys-Thr, indicating that the α-factor prepro-peptide was cleaved at the Ste13 signal cleavage site, as expected. MALDI analysis revealed that the molecular mass of the recombinant UK1 was 10,297.01 Da, close to the calculated mass (10,351.7 Da), and that it has been purified as one component with high purity (Fig. 5). The first peak (5,153.10) represents the doubly protonated form of the protein arising from the MALDI mass spectronic process. All these data indicate that UK1 is not glycosylated, in agreement with the fact that UK1 has no glycosylation site [2].

Dose-Dependent Inhibition of Endothelial Cell Proliferation by UK1

The purified UK1 was assayed for its inhibitory activity on human umbilical endothelial cell proliferation stimulated by several growth factors. With all the growth factors tested, the recombinant UK1 exerted growth inhibitory effects in a dose-dependent manner (Fig. 6). The recombinant UK1 inhibited more effectively VEGF-stimulated proliferation (ED_{50}=20–80 nM), than bFGF- or EGF-stimulated proliferation (ED_{50}=80–360 nM). These results are similar to those obtained from the recombinant UK1 produced by a prokaryotic expression and refolding [16]. This result confirmed that *Pichia*-driven UK1 has inhibitory activity equivalent to *E. coli*-driven UK1.

The above described results suggest that UK1 produced by *Pichia* transformants can be effectively used for the inhibition of angiogenesis. The *Pichia*-driven recombinant kringle 2 domain of tissue-type plasminogen activator has also been shown to be secreted as a properly-folded kringle domain, demonstrated by proper interaction with various ω-amino acid ligands and conformational properties, when studied by differential scanning calorimetry and high-resolution ^{1}H-NMR [21]. *Pichia* expression of bovine β-lactoglobulin

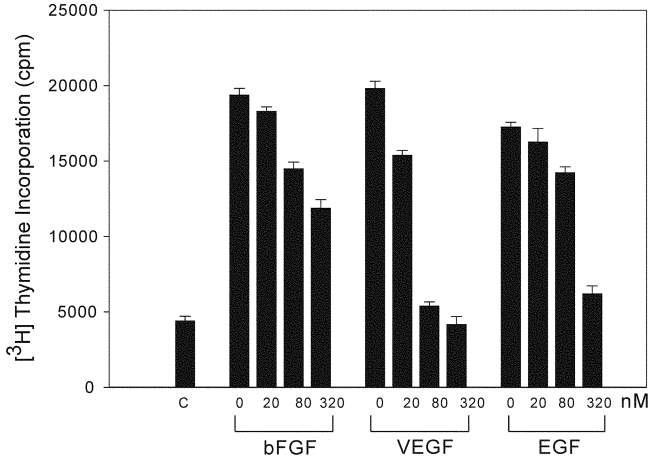

Fig. 6. Dose-dependent inhibition of endothelial cell proliferation by UK1.
HUVE cells were cultured with 3 ng/ml bFGF, 10 ng/ml VEGF, or 10 ng/ml EGF with or without various amounts of UK1 for 18 h, followed by [^{3}H]-thymidine incorporation assay. C presents a condition of no growth factor and no inhibitor. Each value represents the mean±SE.

also provided the recombinant protein indistinguishable from those of purified native bovine β-lactoglobulin, when characterized by a number of methods, including CD spectroscopy, guanidine-HCl unfolding, crystallization, and two-dimensional ^{1}H-NMR spectroscopy [17]. Since UK1 has no glycosylation site, *Pichia* expression may provide a more likely native form of UK1. In addition, it provides a large amount of the recombinant UK1. In the previous report [16], it was shown that the concentration of UK1 to have 50% inhibition (ED_{50}) of bovine capillary endothelial cells was about 80 nM, similar to that for angiostatin. Considering that the molecular weight of UK1 is one-third of that of angiostatin, it can efficiently be used for development of antiangiogenic and antitumor agents.

In conclusion, the efficient production of human urokinase kringle domain by secretory expression in *Pichia pastoris* and its simple purification procedure are described. Employing the system described, large amounts of recombinant UK1 can be obtained for several applications. In order to test *in vivo* effect of UK1 in animal tumor models, large-scale production of UK1 by fermentation is currently in progress.

Acknowledgments

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