Biotechnology Letters (2005) 27: 167–171

High level expression of kringle 5 fragment of plasminogen in *Pichia pastoris*

Yufei Zhou[1], Quan Zheng[1], Jin Gao[2] & Jun Gu[1,*]
[1]*National Key Laboratory of Protein Engineering and Plant Gene Engineering, LSC, Peking University, Beijing, P.R. China*
[2]*Molecular Pharmacy Division, Institiution of Biophysics, Chinese Academy of Sciences, Beijing, P.R. China*
**Author for correspondence (Fax: +86-10-6275-6174; E-mail: gj@pku.edu.cn)*

Received 15 October 2004; Revisions requested 9 November 2004; Revisions received 2 December 2004; Accepted 3 December 2004

Key words: anti-proliferation, high expression, *Pichia pastoris*, recombinant kringle 5

Abstract

Angiogensis can be blocked by inhibitors such as endostatin and angiostatin. The kringle 5 fragment of plasminogen also has a potent inhibitory effect on endothelial cell proliferation and leads to the inhibition of angiogenesis. It has promise in anti-angiogenic therapy due to its small size and potent inhibitory effect. Preparation of kringle 5 has been achieved through the proteolysis of native plasminogen and recombinant DNA technology. Bacterially expressed recombinant kringle 5 is mainly insoluble and expressed at low level. The refolding yield is also low. To produce recombinant human kringle 5 in a large quantity, we have genetically modified a strain of *Pichia pastoris*. On methanol induction, this strain expressed and secreted biologically active, recombinant kringle 5. The expression level of the engineered strain in culture reached more than 300 mg l^{-1}. Purification was easily achieved by precipitation, hydrophobic and DEAE ion exchange chromatography. The recovery of recombinant kringle 5 was about 50% after purification. Yeast-expressed kringle 5 has a higher activity in anti-endothelial proliferation than bacterially expressed kringle 5.

Introduction

Angiogenesis is a tightly controlled process by two counter-balancing systems: angiogenic and anti-angiogenic (Bussolino *et al.* 1997). Angiogenesis can be stimulated by factors such as vascular endothelial growth factor (VEGF), and inhibited by variant inhibitors such as angiostatin, endostatin and pigment epithelium-derived factor (PE DF) (Dawson *et al.* 1999, Jimenez & Volpert 2001). Plasminogen kringle 5 (K5), a fragment of plasminogen, has been identified to be a potent angiogenic inhibitor (Cao *et al.* 1997). It induces apoptosis and causes arrest of the cell cycle in proliferating endothelial cells (Lu *et al.* 1999). The anti-angiogenic effect of K5 on vascular cells is involved in down-regulation of endogenous VEGF and up-regulation of PEDF (Dawson *et al.* 1999, Jimenez and Volpert 2001). Intravitreal injection of recombinant K5 prevents the development of, and arrests the progression of, ischemia-induced retinal neovascularization in a rat model (Zhang *et al.* 2001), which implicates that K5 has a potential therapeutic significance in angiogenesis related diseases.

Many efforts have been made to prepare K5 including the proteolysis of plasminogen and recombinant DNA technologies (Cao *et al.* 1997, Lu *et al.* 1999, Stathakis *et al.* 1999, Zhang *et al.* 2001). K5 has been successfully produced in *E. coli* either as a protein secreted into periplasm space or as an insoluble protein in inclusion body. However, the expression of K5 in bacteria only gives low yield of soluble and relatively high level of insoluble protein. In general, recovery of soluble protein from *in vitro* renaturation or refolding not only needs complicated protocols and lengthy times but also has a low efficiency. The treatment of animal models for anti-angiogensis with angiogenic inhibitors, including K5, requires a high dose and

multiple injections, which needs a large quantity of proteins. Therefore, efficient production of K5 with low cost is desirable for the future application in the therapy of anti-angiogensis.

Pichia pastoris has been widely used to produce foreign proteins. As a eukaryote, it has many of the advantages of higher eukaryotic expression systems such as protein processing, protein refolding and post-translational modification, while being as easy to manipulate as *E. coli*. Besides, it has the added advantage of 10- to 100-fold higher heterologous protein expression levels. Heterologous expression in *Pichia* can be either intracellular or secreted (Romanos *et al*. 1992). Here we report a genetically engineered *Pichia* strain producing a high level of recombinant K5. It expressed secreted K5 to over 300 mg l^{-1} in culture. Yeast-expressed K5 had a higher biological activity than bacterially expressed K5. It shows a potential to be used in industrial production and clinical application.

Materials and methods

Materials

The plasmid pET21b-K5 was kindly provided by Dr Jianxin Ma. A Pichia expression kit and plasmid pPIC9K were purchased from invitrogen (Houston, TX). DEAE sepharose fast flow and Phenyl Sepharose 6 Fast Flow were the products from Pharmin (Piscataway, NJ). HUVEC cells were purchased from Cancer Research Institute of Medical School Hospital of Peking University.

Preparation of K5 in E. coli

Recombinant K5 (rK5) expressed in *Escherichia coli* was prepared as described previously (Zhang *et al*. 2001).

Generation of P. pastoris *producing recombinant K5*

The plasmid pET21b-K5 was used as a template to amplify human plasminogen kringle (K5) by PCR with the forward primer: 5′-CTTCCTCGAG AAAAGAGATGTAGAGAC-3′ and reverse primer: 5′-TAGCGAATTCTTATCAGGCACA CTGAGG-3′. The PCR product contained a cDNA sequence coding 92 amino acids of K5: KRDVE TPSEEDCMFGNGKGYRGKRATTVTGTP CQDWAAQEPHRHSIFTPETNPRAGLEKN YCRNPDGDVGGPWCYTTNPRKLYDYC DVPQCA. The cDNA was then sub-cloned into pPIC9K vector at the sites of *Xho*I and *Eco*RI to form pPIC9K-K5.

The pPIC9K-K5 was linearized by *Sal*I and transformed into *Pichia* GS115 by electroporation. *Pichia* integrants were selected according to the instruction from the manufacturer (Invitrogen, USA). The selected integrants were seeded on plates at 5×10^5/plate with YPD medium containing G418 at 0.25, 0.5, 1, 2, 4 mg ml^{-1}. Strains with high expression efficiency were determined by screening secreted K5 using Tricine SDS-PAGE. The integration of K5 was confirmed by PCR.

Expression of recombinant K5 by the engineered P. pastoris *strain*

A single clone was grown in 20 ml YNB overnight at 30 °C, and then inoculated at 2% (v/v) into 200 ml BMGY, and grown at 30 °C with shaking of 250 rpm. Cells were collected by centrifugation at 3000 *g* when the OD_{600} of the culture reached 2.5. The collected cells were re-suspended in 500 ml BMMY and cultured for another 4 d at 30 °C with shaking. Methanol was added daily to give a final concentration of 0.5% during the culture. The supernatant of cell culture was collected by centrifugation at 6000 *g* for 10 min after 4 days' culture, and used for the determination and purification of secreted K5.

Purification of recombinant K5 expressed by P. pastoris

Ammonium sulfate was added to the above supernatant. The final concentration of ammonium sulfate was 85%. The precipitate after centrifugation was re-suspended in 50 ml 0.05 M PBS (pH 7.4, with 1.7 M ammonium sulfate), and then applied to a Phenyl Sepharose 6 Fast Flow column (15×60 mm) equilibrated with 0.05 M PBS (pH 7.4, with 1.7 M ammonium sulfate). The column was eluted with the equilibration buffer (0.05 M PBS, pH 7.4). The eluent containing K5 was collected and adjusted to pH 8 with phosphate buffer pH 8 and applied to the

following purification. Column was equilibrated with phosphate buffer (pH 8) before the sample was applied to a DEAE Sepharose Fast Flow column, and then eluted with phosphate buffer (pH 8, with 1 M NaCl). The elution fractions were collected and identified by 10% Tricine SDS-PAGE.

Anti-endothelial cell proliferation assay

Monolayers of HUVEC cells were maintained in medium 1640 containing 10% FBS and bFGF (3 ng ml^{-1}). Cells were dispersed in a 0.05% trypsin solution and re-suspended with medium 1640 containing 10% FBS after collection. Approximately 10^5 cells were seeded into each fibronectin (1 ng ml^{-1}) coated well of a 12-well plate in 1 ml medium. The medium was replaced by 0.5 ml of fresh medium 1640 with 5% FBS after 24 h culture at 37 °C. Different concentrations of K5 were added to each well followed by adding 0.5 ml medium 1640 with 5% FBS and bFGF (2 ng ml^{-1}) after 30 min of incubation at 37 °C. Cells were harvested after incubation at 37 °C for 72 h, and finally subjected to MTT assay.

Results

Expression of a high level of recombinant K5

The constructed plasmid pPIC9K-K5 was used to transform *P. pastoris* to generate genetically modified strain for the production of kringle 5. Eight integrants were identified and tested for the secretion of K5. All of them produced secreted recombinant K5 on the induction of methanol (Figure 1a). Secreted K5 had a molecular size of about 10 kDa that is consistent with the molecular weight deduced from its amino acid sequence. The level of secreted K5 was increased with time (Figure 1b). The maximal expression level appeared at the 5th day. The amount of secreted K5 was determined by comparison with lysozyme standard in SDS-PAGE as shown in Figure 1c. Estimated level of secreted K5 was more than 300 mg l^{-1}.

Purification of yeast-expressed K5

One of the advantages for secreted proteins is an easy purifcation. The supernatant from the culture of the engineered strain was concentrated by

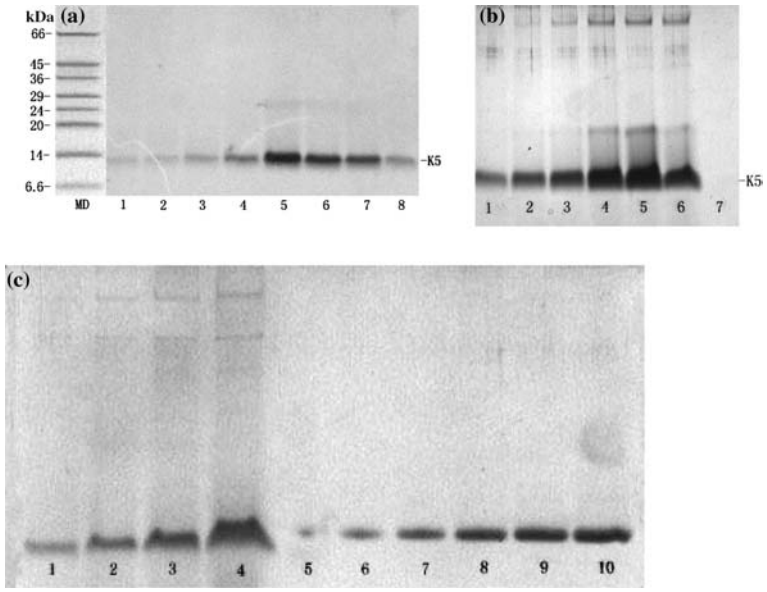

Fig. 1. Induction of K5 in genetically modified *P. pastoris* strains. (a) Secretion of K5 from 8 integrants (lane 1–8) was determined by Tricine SDS-PAGE. (b) Ten μl of supernatant from the selected transgenic GS115 strain cultured under methanol induction for 1 d (lane 1), 2 d (lane 2), 3 d (lane 3), 4 d (lane 4), 5 d (lane 5) and 6 d (lane 6), respectively, was resolved on Tricine SDS-PAGE. Lane 7 was the supernatant without induction. (c) One μl (lane 1), 2 μl (lane 2), 5 μl (lane 3) and 10 μl (lane 4) supernatant of transgenic GS115 strain, and 0.1 μg (lane 5), 0.2 μg (lane 6), 0.5 μg (lane 7), 1 μg (lane 8). Two μg (lane 9) and 4 μg (lane 10) of lysozyme as standard, were resolved on Tricine SDS-PAGE.

precipitation with ammonium sulfate. The chromatography with Phenyl Sepharose was used first to remove the salt from the sample. The eluent containing K5 was then used for further purification by DEAE Sepharose Fast Flow column. As seen in Figure 2, purified K5 displayed one single band in SDS-PAGE (Figure 2a). The purity was further confirmed by RP-HPLC (Figure 2b). The yield of K5 was about 150 mg l^{-1} after purification.

Yeast-expressed K5 has a higher activity than bacterially expressed K5 in anti-endothelial cell proliferation

K5 has been previously expressed in *E. coli* as a soluble periplasmic form (Zhang *et al.* 2001). The bacterially expressed K5 was about 16 kDa in molecular size (Figure 3a, lane 1). It is bigger than predicated from its amino acid sequence, including hexahistidine, which should be about 12 kDa. Yeast-expressed K5 appeared at its predicted molecular size of about 10 kDa (Figure 3a. lane 3). As shown in Figure 3b, the yeast-expressed K5 had a more potent activity of anti-endothelial proliferation than bacterially expressed K5.

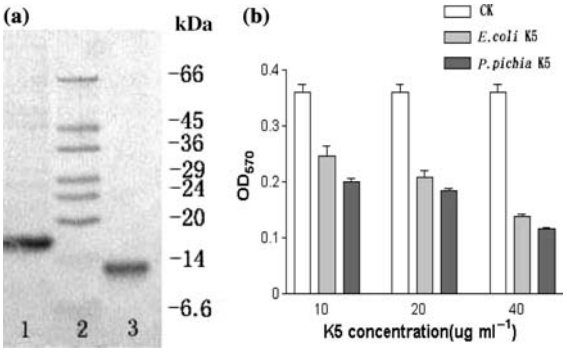

Fig. 3. Comparison of yeast-expressed K5 with bacterially expressed K5. (a) Bacterially expressed K5 (lane 1) and yeast-expressed K5 (lane 3) were resolved on Tricine SDS-PAGE. (b) MTT assay for anti-endothelial cell proliferation by bacterially expressed K5 and yeast-expressed K5. Each bar represented value from 8 parallel experiments and was analysed by Prism 3.03 program. White bar, cells treated with PBS; gray bar, cells treated with bacterially expressed K5; dark bar, cells treated with yeast-expressed K5.

Discussion

K5 has been expressed in *E. coli* (Zhang *et al.* 2001). Although it secretes into periplasm and is soluble, the expression level is low at about 20–30 mg l^{-1} culture. It usually deposits as an inclusion body when expressed in *E. coli*, and refolding yield from the inclusion body is very low (Cao *et al.* 1997, Lu *et al.* 2001, 2002). Bacterially expressed K5 is often fused to the hexahistidine tag, which makes it easy to be purified though the cost of an affinity resin used for the purification of K5 is expensive. These issues raise the concern of using bacterial expression system for the production of K5.

Pichia pastoris is known for its high expression of foreign genes. It also secretes the products with fewer and lower levels of endogenous host proteins into the medium, which greatly favors the purification of target protein. We used *P. pastoris* as K5 expression system and successfully generated a genetically modified strain that secreted K5 to more than 300 mg l^{-1} (Figure 1). Another

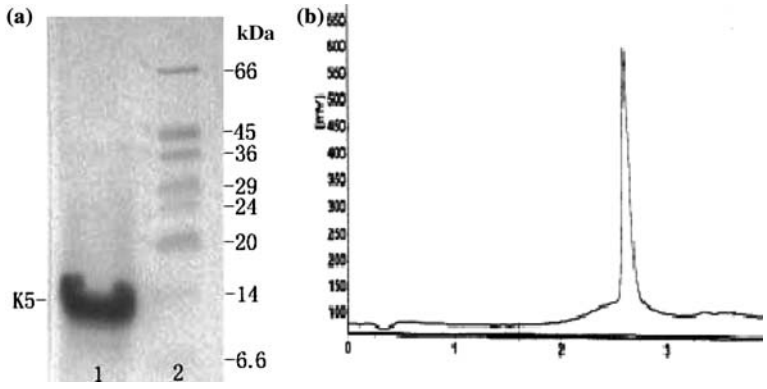

Fig. 2. Purification of K5. (a) Purified K5 was resolved on Tricine SDS-PAGE. Lane 1: purified K5; lane 2: molecular weight marker. (b) Analysis of purified K5 by reversed-phase hydrophobic HPLC.

P. pastoris-expressed K5 system has been reported recently (Zhu *et al*. 2003). However, expression in this system was only 30 mg l^{-1} which is a tenth of ours. The high level expression could have resulted from the multiple copies of K5 gene integrated into yeast genome. We have used high concentration of antibiotic to select transfected GS115 strain which could lead to the selected strain having multiple copies of the transgene. To identify if there are multiple copies of transgene integrated into host chromosome, we performed a Southern Blot assay. However, multiple copies were not seen (data not shown). Alternatively, it is also possible that the integration position of transgene causes the difference in expression level.

The bacterially expressed K5 has a molecular size higher than expected. It should be about 12 kDa, instead of 16 kDa in SDS-PAGE according to its amino acid sequence (Figure 3). Yeast-expressed K5 displays the molecular size consistent with its amino acid sequence. This could reflect that K5 is modified differently when expressed in bacteria and yeast. To address if the different modification of K5 has the effect on its biological function, we tested their activity in anti-endothelial proliferation. The results reveal that yeast-expressed K5 has a higher activity than bacterially expressed K5 (Figure 3). It indicates that that yeast-expressed K5 may hold structural configuration more close to the native form comparing to the bacterially expressed K5.

In conclusion, we report a genetically engineered *P. pastoris* strain producing high level of native kringle 5. Yeast-expressed K5 had a more potent activity in anti-endothelial proliferation than bacterially expressed K5. With the properties of higher activity, intact form, easy purification for K5, this strain shows a prospect for a large amount of production of K5 in industrial and clinical application.

References

Bussolino F, Mantovani A, Persico G (1997) Molecular mechanism of blood vessel formation. *Trends Biochem. Sci.* **22**: 251–256.

Cao YH, Chen A, An SSA, Ji RW, Davidson D, Cao YM, Linas M (1997) Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth. *J. Biol. Chem.* **272**: 22924–22928.

Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, Bouck NP (1999) Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* **285**: 245–248.

Jimenez B, Volpert OV (2001) Mechanistic insights on the inhibition of tumor angiogenesis [review] *J. Molec. Med.* **78**: 663–672.

Lu H, Dhanna M, Volk R, Waterman MJF, Ramchandran R, Knebelmann B, Segal M, Sukhantme VP (1999) Kringle 5 causes cell cycle arrest and apoptosis to endothelial cells. *Biochem. Biophys. Res. Commun.* **258**: 668–673.

Lu H, Yuan H, Li Y (2002) Expression of human plasminogen kringle 5 as fusion protein with truncated hIFNgamma gene in *Escherichia coli*. *J. Biotechnol.* **94**: 277–285.

Lu H, Zhang H, Wang Q, Yuan H, He W, Zhao Z, Li Y (2001) Purification, refolding of hybrid hIFN gamma-kringle 5 expressed in *Escherichia coli*. *Curr. Microbiol.* **42**: 211–216.

Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. *Yeast* **8**: 423–488.

Stathakis P, Lay AJ, Fitzgerald M, Schlieker C, Matthias LJ, Hogg PJ (1999) Angiostatin formation involves disulfide bond reduction and proteolysis in kringle 5 of plasmin. *J. Biol. Chem.* **274**: 8910–8916.

Zhang D, Kanfman PL, Gao G, Saunders RA, Ma JX (2001) Intravitreal injection of plasminogen kringle 5, an endogenous angiogenic inhibitor, arrests retinal neovascularization in rats. *Diabetologia* **44**: 757–765.

Zhu M, Cheng J, Hua ZC. (2003) Expression of kringle 5 domain of human plasminogen in *Pichia pastoris*. *Prep. Biochem. Biotechnol.* **33**: 269–281.