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Activity and Integrating Expression of Human Endostatin Produced by *Pichia pastoris*

Research Article

Chongbi Li[1,*], Minghai Zhou[2], Hongbin Cui[3], Zhan Wang[3]

[1]The Center of Biopharmaceutical Research and Development of Zhaoqing University, 526061, China (PR)
[2]Institute of Microbiology, Institute of Microbiology, Academia Sinica, Taiwan
[3]Gene Science Pharmasutical Company in Beijing, 100000

***Correspondence**: Chongbi Li, Donggang, Zhaoqing City, Guangdong Province, Biopharmaceutical Research & Development Center of Zhaoqing University, 526061, China; Tel: 0086-0758-2716359; Fax: 0086-0758-2776882; Email: lchongbi@yahoo.com

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Abbreviations: alcohol oxidase I, (*AOX1*); chorioallantoic membrane, (CAM); Hen's Egg Test – Chorioallantoic Membrane, (HETCAM); methyl cellulose, (MC); phosphate buffered saline, (PBS); transcription terminator, (TT); wet cell weights, (wcw)

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Summary

Heterologous expression in *Pichia pastoris* has many of the advantages of eukaryotic expression, proper folding and disulfide bond formation, glycosylation, and secretion. Contrary to other eukaryotic systems, protein production from *P. pastoris* occurs in simple minimal defined media making this system attractive for production of expressed proteins for purification. Endostatin is a potent and specific antiangiogenic protein capable of inhibiting the growth of murine and xenotransplanted human tumors. Thus far, however, recombinant endostatin prepared from *Escherichia coli* has been insoluble after purification and therefore inappropriate for clinical settings. In this study, human endostatin gene was integrated into the chromosome of host *pichia pastoris* by using the yeast inserted plasmid pPICZaA-endo including human endostatin gene, native Saccharomyces cerevisiae a-factor secretion signal, zeocin resistant gene and the AOX1 promoter and transcription terminator (TT) of *pichia pastoris* electroporation. The recombinant clones were then selected by the plates containing antibiocin-zeocin (100ug/ml), and finally one highly expressed clone was selected by PCR, SDS-PAGE and Western-blot. The yield of expressed endostatin from *P. pastoris* depended critically on growth conditions, and attainment of high cell densities by fermentation had been shown to improve protein yields up to 15mg/L estimated. Moreover, the protein was easily purified by using a heparin-agarose column. A strengthened antiangiogenic activity in vivo has been identified by a novel and simple CAM (chorioallantoic membrane) technique.

I. Introduction

Endostatin (ES) is a 20-kDa fragment cleaved from a collagen XVIII COOH terminus that inhibits endothelial cell proliferation, migration, invasion, and tube formation (O'Reilly et al, 1997) and that has shown antiangiogenic and antitumor effects in animal models (Dhanabal et al, 1999a,b). It specifically binds to neovascular endothelial cells through the integrin receptors $\alpha_5\beta_1$ and $\alpha_V\beta_3$ (Wickström et al, 2002). These results strongly suggest that ES has a unique ability to target neovascular endothelial cells and therefore may be useful in antiangiogenic therapy. Although the mechanism by which ES suppresses angiogenesis in a tumor-specific manner generally remains unclear, it has been shown that ES induces endothelial cell apoptosis and inhibits endothelial migration (Dhanabal et al, 1999b). In previous studies, soluble recombinant protein expressed in yeast needed about 15 mg/L to inhibit the proliferation and migration of endothelial cells in response to stimulation by basic fibroblast growth factor (Dhanabal et al, 1999a). However, the relatively low yield of expression systems thus far has made it difficult to produce ES in quantities sufficient for extensive clinical evaluation. There is therefore a great need to increase the yield and to reduce the cost of the production of recombinant ES that is suitable for clinical use. *Pichia pastoris* yeast is a highly successful system for production of a wide variety of recombinant proteins that are not easily obtained from *E. coli* and it can also be grown on minimal nutrients. Moreover, the proteins produced can be correctly folded into native states and with full activity from *P. pastoris* yeast. Additionally, the most important aspect of the *Pichia pastoris* yeast

expression system is that it contains a promoter derived from the alcohol oxidase I (*AOX1*) gene of *P.pastoris*. This promoter is uniquely suited for the controlled expression of foreign genes and the strong preference of *P. pastoris* for respiratory growth, a key physiological trait that greatly facilitates its culturing at high-cell densities relative to fermentative yeasts.

In order to boost the yields of ES expression, *Pichia pastoris* was used as expressing host to express human ES and by further fermentation of the initial yield. CAM (chorioallantoic membrane) technique was adopted for determining antiangiogenic activity of recombinant ES.

II. Materials and Methods

A. Plasmid, bacteria and chemical reagents

The *Pichia* Expression System has been engineered to make it as easy to use as bacterial systems. It offers the following advantages: A simple, convenient method for rapidly preparing and transforming competent *Pichia pastoris* cells eliminating the tedious and time-consuming preparation of spheroplasts Vectors containing the Zeocin™ resistance gene to allow direct selection of transformed cells.

B. Cloning and transformation

Zeocin™-resistant pPICZ a *Pichia* vector (Invitrogen) containing ES was linearized at a unique restriction site in the *AOX1* region. Competent GS115 *Pichia pastoris* cells (stored in our lab) were prepared and transformed by electroporation (electroporation apparatus from Biorad Corp.) with the linearized constructs according to the instructions of EasySelect™ Pichia Expression kit (Invitrogen). Transformants were plated on the appropriate medium (YPD, YPDS, BMGY, BMMY or YPDS plus 100 μg/ml Zeocin). Colonies were selected from each plate and patched onto YPDS (Zeocin 100ug/ml) plates containing X-gal for two days at 30 °C with shaking (250 rpm). GS115 was used as a control (bottom right patch). One hundred percent of the pPICZ a /*lac*Z colonies called pPICZ a-endo were blue and therefore contain the *lac*Z gene. Otherwise, C-terminal polyhistidine (6xHis) sequence would facilitate an efficient detection and rapid purification of recombinant ES. The positive clone with *AOX1* promoter for high-level, methanol-induced expression was then chosen. All reagents used including restriction enzymes, Tag polymerase, RNase, SDS and some other chemical reagents were the highest grade possible from Bolimeger, Biotech, Sigma and Beijing Chemical Reagents Corp.

C. Screening by PCR and oligonucleotide synthesis

In order to screen the correct positive *P. pastoris* clonies, some primers were designed beforehand according to the sequence of 5′-end and 3′end of AOX1 of *P. pastoris* chromosome and rhES gene and synthesized at Sangon Corp in Shanghai including ES upstream primer-5′-GAGAAAAGACACAGCCACCGCGACTTC-3′, downstream primer 5′-TGCGAATTCTTACTACTTGGAGGCAGTCAT-3′, upstream primer of *P. pastoris* AOX1 5′-GACTGGTTCCAATTGACAAGC-3′ and its downstream primer 5′-GCAAATGGCATTCTGACATCC-3′. The colonies were able to grow in yeast. The chromosome of recombinant *P. pastoris* would be isolated by methods previously described (Sambrook et al, 1989). The recombinant DNA was analyzed by PCR using the primers mentioned above.

D. Fermentation protocol for recombinant Pichia pastoris

In order to ferment recombinant *Pichia pastoris* on large scale, firstly, shake flask cultures (1 L starting volume) were inoculated from a 10 ml culture of transformant *P. pastoris* that was grown from a frozen stock overnight in YPD with shaking at 30 °C and grown in either YPD or BMMY for two days at 30 °C with shaking of 250 rpm (revolution per minute). Cells were harvested and resuspended in 500 mL of the same growth medium but containing 2% methanol instead of glycerol. Induction periods were typically 48 h. The protein expressions of human ES in *Pichia pastori* were detected by Western analysis (Sambrook et al, 1989). In brief, the protein samples (50 μg/lane) were resolved by 8 and 12% SDS-PAGE for endostatin, under denaturing and reducing conditions and transferred to nitrocellulose membrane. Rabbit anti-ES antibody (prepared by GSPC Lab.) as well as secondary antibodies including a dig-linked species-specific sheep anti-rabbit IgG antibody (Bolimeger) were used for immunoblotting. Western blotting reagents were also bought from Bolimeger, a NBT/BCIP system was used for the blot development (Sambrook et al, 1989). The images of signals were electronically digitalized by scanning, and the intensity of images were quantitated by Image Quant version 1.2 (Molecular Dynamics, Sunnyvale, CA).

The fermentation media was sterilized in the fermentor vessel by autoclaving, and after slow cooling was allowed to oxygenate and the pH was slowly adjusted by addition of the base solution. Recombinant *P. pastoris* by fermentation was carried out in a Biostat 5 L fermentor (B.Braun Corp), equipped with a 1.25 L bioreactor. A 20 ml culture of *P. pastoris* was grown from a frozen stock overnight in BMM with shaking at 30 °C. The culture (Zeocin, 100ug/ml) was used to inoculate a 200 mL culture (no Zeocin) of YPD in a 500 mL Erlenmeyer flask and was grown for 24h shaking at 250rpm, at 30°C. The pelleted cells were resuspended in 2L BMGY culture at 30 °C.The pH was maintained at 5.5 by addition of the KOH and NaOH base mixture with a maximal flow rate of 20 mL/min. During the initial growth period, the *P. pastoris* cells used the glycerol that is present in the basal salts medium, and this batch growth phase typically lasted less than 15h. Towards the end of the batch growth phase, the cells started to utilize O_2 at a high rate requiring pure O_2 supplementation to maintain the dissolved O_2 levels at 30%. After 15h of fermentation, 50% glycerol (100g) was fed, at a rate of 20 mL/min over 20 h. The cells reached final wet cell weights (wcw) of 240 g/L. At this point, glycerol supplement would be stopped. Towards the end of the batch growth phase, when the cells started to utilize O_2 at a high rate requiring pureO_2 supplementation to maintain the dissolved O_2 levels at 30%, pH value was rising and the glycerol in solution was exhausted, methanol induction was initiated with a solution of 24% methanol. The flow rate of methanol was increased from 0.36mL/h to 3.6mL/h and the concentration of methanol would increase up to 70% until the end of the induction phase, which terminated after 120h. The cells were separated from the culture supernatant by centrifugation at 4200g for 1h in a Becman centrifuge. A solution of 0.5 M EDTA was added to the culture supernatant to a final concentration of 10 mM. The culture supernatant was stored at -70°C until protein purification.

G. Purification for recombinant ES

After fermentation, the supernatant was collected with low-speed centrifugation. The coarse protein was isolated with ammonium sulfate (70%) precipitated. The pellet was collected with 1500g centrifugation for 15min, followed by dialysis with10mM Tris buffer (pH7.4) at 4℃ for 8 h. After the protein pulled out salt dissolving with 10mM Tris (pH7.4), the lysate was then applied to a heparin-column (QIAGEN, Valencia, CA).

Endostatin was eluted with 10mM Tris (pH 7.4) by a gradient of NaCl from 0-1M. The fractions at the concentrations of 0.2-0.6M eluted fluid were collected. The fractions containing ES protein were then concentrated and analyzed by SDS-PAGE previously described (Sambrook et al, 1989).

H. Sequencing analysis for ES

The purified protein would run on an SDS-PAGE gel and then electroblot to a PVDF membrane, stain with Coomassie and cut out the band of ES. Determining the sequence of amino acids in recombinant human ES was carried out by automated techniques (The Protein Facility utilizes a Perkin Elmer Applied Biosystems 491A) in Peking University. N-terminal six amino acids sequences would be determined.

I. Antiangiogenic activity for recombinant ES *in vitro*

The Hen's Egg Test – Chorioallantoic Membrane (HETCAM) is an established model system to evaluate many different parameters of tumor growth (Chambers et al, 1992) and antineoplastic drug screening (Brooks et al, 1994). The CAM model system conveniently and inexpensively reproduces many of the characteristics of tumors *in vivo*, such as tumor mass formation, angiogenesis or neovascularization, infiltrative growth and metastasation. This model presents a series of advantages over conventional animal models, including simplicity, low cost, and natural immunodeficiency of the chick embryo and the CAM which makes them good hosts for a great variety of cells and tissues. The vascular density of the CAM would be taken as a criterion to evaluate the activity of recombinant human ES. The method was modified according to an established model system in order to detect the activity of ES. Concretely operation is as below.

For getting a film to be used assaying the activity of ES, a drop of 0.5% methyl cellulose (MC) would be added in a non-taint pan and blown dry to prepare a film. Then a drop of bFGF (basic fibroblast growth factor) about 10ul(1ug/ml), rhES 10 μl and 20 μl (1mg/ml) as well as phosphate buffered saline (PBS) 20 μl which was used as a negative control would be dropped on the film, respectively. Then the film was blown dry again. Fertilized chicken eggs were obtained from a local hatchery and were incubated in a forced-air incubator (HJQ-F160, Shanghai) with an automatic turner, at 37ºC and ~80% relative humidity until incubation day 7. Viability of the embryos was assessed daily by candling. Then surface of the egg was sterilized with 70% of ethanol and a 2-cm diameter wide window was opened aseptically carefully in the flat pole of the eggshell with an electric drill. The exposed surface of the dermic sheet on the floor of the air sac was wetted with a few drops of sterile PBS, then carefully punctured with fine sterile watchmaker's forceps and removed to show the underlying CAM. Care was taken at this point to avoid any injury to the highly vascularized CAM. Then fine different medicine-MC films prepared were carefully laid on the sections of CAM neovascularization and then removing it immediately. Following inoculation, the window was closed with sterile, glass cover slip and sealed in place with heat-melted glue. The embryo was returned to the incubator in an upright position, without turning, and remained there for seven additional days, with daily monitoring. At the time of harvest (~incubation days 17), the embryo was killed by hypothermia. The eggshell was carefully broken at one pole and the egg was fixed *in toto* in 1.5 ml solutions of acetone and methanol (1:1) for 20 min. The CAM was then dissected out with scissors, photographed by transillumination in a Stemi SV11 stereomicroscope (Zeiss, Germany).

III. Results

A. Screening for inserting integration

The clones grew on the plates containing antibiotics were detected by PCR. And the products by PCR run electrophoresis of agarose, and the clones containing 2.2Kb and 1.2 Kb fragments could be amplified by PCR with primers of AOX1 5 and 3-end well as 552bp fragment could be amplified by PCR with primers of human ES gene 5 and 3-end were selected as positive clones (**Figure 1**).

B. Expression and detection of recombinant ES protein

Our small-scale expression screening procedure by PCR identified a bacterial colony possessing the high expression efficiency, from a cohort of 20 -transformed colonies. This clone termed GSSpICZa A1 was subsequently used for large-scale (1 liter) expression and fermentation. In our protocol, when 10 μg of protein were analyzed by silver-staining SDS-PAGE, a significant protein band of M_r 18.3kD corresponding to ES was seen after induction at days 2 and the highest expression was at 4 days (**Figure 2**). And a blotting band also showed on the western-blot membrane (**Figure 3**).

C. Fermentation of recombinant P. pastoris and purification of rhES

Fermentation conditions with high protein expression were obtained through the fermentation practice. After fermentation under optimized conditions of pH, temperature and dissolved O_2 levels, the culture supernatant was carried out protein purification with ammonium sulfate (70%) precipitated and running a heparin-column. The purifity of ES isolated was up to 85%, and the amount of the protein was about 15mg/L (**Figure 4**).

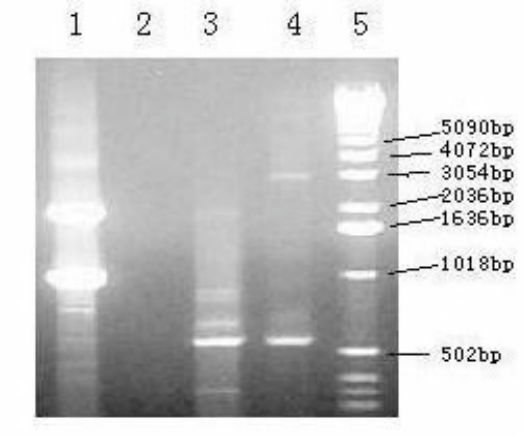

Figure 1. PCR amplification. 1. Amplying fragment of inserted chromosome (primer: AOX1); 2: negative control; 3: endostatin gene from inserted chromosome (primer: endostatin 5'and 3'); 4: pPICZaA-endo(primer:endostatin 5'and3'); 5: marker.

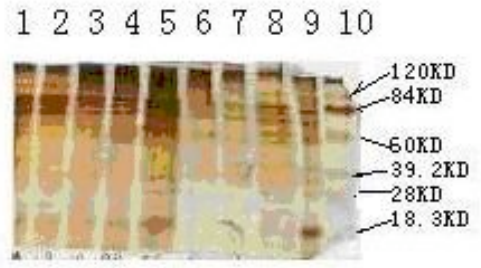

Figure 2. Silver-strained SDS-PAGE of induced Endostatin expressed in P. pastoris. **1-5**. After being induced representing engineered GSSPICZaA days 1 3, 4, 5, 6, 7, respectively; **6**: GSS115; **7**: 4th day (GSSPICZaA9); **8**: 4th day (GSSPICZaA25); **9**: E.coli cell with pET-endo; 10: protein marker.

Figure 3. Western-blot. **1-9**: corresponding lanes with **Figure 2.**

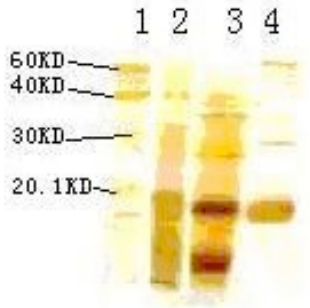

Figure 4. SDS-PAGE from rhEndostatin purification **1**.protein marker **2**. engineered E.coli with pET-endo; **3**.the sample with ammonium sulphate (70%) precipitated; **4**. after purification.

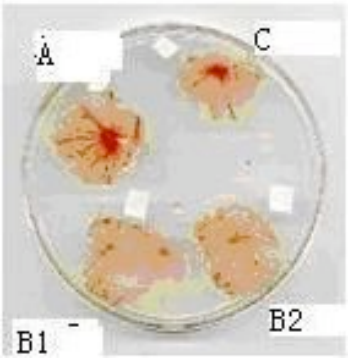

Figure 5. CAM assay. **A**: CAM with bFGf; C: negative control; **B1**: CAM with rhEndostatin (20 μg) and **B2**: CAM with rhEndostatin (10 μg protein).

D. Protein sequencing

The result by N-terminal from 1 to 6 amino acids sequencing showed that of that either the first amino acid histidine of rhES at N-terminal had been substituted for arginine or the former 4 amino acids of rhES at N-terminal had been deleted. It indicated that endostatin may have two forms after recombination

E. Antiangiogenic activity of recombinant endostatin

The result from CAM test showed that bFGFcould promote a plethora of vascular endothelial cells, and rhES could inhibit the CAM neovascularization effectively. In our experiment, it showed that 10 and 20ug ES resulted in a 90% and 70% inhibition (**Figure 5**).

IV. Discussion

In this report we chose to produce human ES in *P. pastoris* by fermentation because higher protein yields at a lower cost than from animal cells was expected. In the *P. pastoris* expression system, the main characteristic is that the genes of interest are expressed under the control of the *Pichia* alcoholoxidase 1 (AOX1) promoter. This promoter is tightly regulated and induced by methanol which also serves as carbon source during induction. Expression level s in milligrams of purified ES expressed has been obtained. As a yeast, *P. pastoris* is a single-celled microorganism that is easy to manipulate and culture. However, it is also a eukaryote and capable of many of the post-translational modifications performed by higher eukaryotic cells such as proteolytic processing, folding and disulfide bond formation. Thus, many proteins that end up as inactive inclusion bodies in bacterial systems are produced as biologically active molecules in *P. pastoris* (Wood and Komives, 1999; Ferreras et al, 2000). Additionally, *P. pastoris* secretes only low levels of endogenous proteins because the strain used in this study was of the proteinase K deleted phenotype, and its culture medium contains no added proteins. Therefore, the secreted heterologous protein in the culture supernatant was facilitated by protein purification. Though ES expression in *P. pastoris* had been reported before, the expression yields at 10mg/L were relatively low (Volk et al, 1999). In our experiment, yields of protein produced in *P. pastoris* grown in fermentation were much higher culture than in shake flasks reported previously (Jahic et al, 2003). Therefore, the *Pichia* Expression System produces high yields of recombinant ES protein that can be recognized in credible way for meeting clinic needs.

In the experiment, we also found that rhES was not stable and could be easily degraded. Sequencing results indicated that 6 amino acids in the N-terminal of rhES could be altered or deleted depending on ES sensitivity to some proteinases in cells (Ding et al, 1998). However, purified rhES still retained its anti-angiogenic activity. This implies that the active site of ES is not its' N-terminal.

The CAM model system conveniently and inexpensively reproduces many of the characteristics of tumors *in vivo*, such as tumor mass formation,

angiogenesis or neovascularization, infiltrative growth and metastasation. This model presents a series of advantages over conventional animal models, including simplicity, low cost, and natural immunodeficiency of the chick embryo and the CAM which makes them good hosts for a great variety of cells and tissues. In our test, we chose a slightly less rigid mounting medium, methyl cellulose (Sigma) for orienting young embryos (less than 10 h old) to grease proof paper as an experimental film and obtain ing good results.

The results obtained with different medicine-MC films prepared onto the chorioallantoic membrane of the embryo suggests that the chick opens possibilities to test medicines on a large scale and at a low cost in a model quite similar to the *in vivo* situation. This novel and simple method can be suggested to be used in laboratory study for anti-angiogenesis.

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Chongbi Li