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ORIGINAL PAPER

Constitutive expression of human angiostatin in *Pichia pastoris* by high-density cell culture

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Abstract A high-density cell culture method to produce human angiostatin has been successfully established by constitutive expression of the protein in *Pichia pastoris*. The fermentation was carried out in a 20 l bioreactor with a 10 l working volume, using a high-density cell culture method by continuously feeding with 50% glycerol–0.8% PTM4 to the growing culture for 60 h at 30°C. Dissolved oxygen level was maintained at 25–30% and pH was controlled at 5 by the addition of 7 M NH_4OH. Angiostatin was constitutively expressed during the fermentation by linking its expression to the *P. pastoris* constitutive GAP promoter (pGAP). But after 36 h of fermentation, the peak biomass growth was 305 as measured by absorption of 600 nm, while the peak angiostatin expression was 176 mg/l. Similar to the product expressed from inducible system [24], angiostatin produced from constitutive system also inhibited the angiogenesis on the CAM and suppressed the growth of B16 melanoma in C57BL/6J mouse. The above results suggest that GAP promoter is more efficient than AOX1 promoter for the expression of angiostatin in *P. pastoris* by shake flask culture or high-density cell fermentation and is likely to be an alternative to AOX1 promoter in large-scale expression of angiostatin and other heterologous proteins.

Keywords Angiostatin · Constitutive expression · *Pichia pastoris* · High-density cell culture · Bioactivity

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Introduction

The methylotrophic yeast *Pichia pastoris* is an efficient host for expression and secretion of heterologous proteins [2, 10]. It can grow to very high cell densities (40–50% v/v) and secrete recombinant proteins on minimal medium [18, 19]. Also, it performs many posttranslational modifications of the higher eukaryotes [13]. But the most important feature of *P. pastoris* is the existence of a strong and tightly regulated promoter from the alcohol (methanol) oxidase I (AOX1) gene [3]. The methanol oxidase (AOX1) promoter has been chosen to construct expression vectors for production of a variety of recombinant proteins in *P. pastoris* [16]. The gene expressing the methanol oxidase enzyme is tightly regulated and is not expressed unless induced. However, the expression of this enzyme aids cell proliferation when glucose or glycerol is used as sole carbon source and is extremely high in the presence of methanol [20]. The inducible expression system is especially effective for gene expression whose product is toxic to the host. But methanol is a toxic substance and easy to cause environment pollution in

production. In addition, it is inconvenient to switch from glycerol to methanol during fermentation [21].

Glyceraldehyde-3-phosphate dehydrogenase (GAP) is a key enzyme in glycolysis; its promoter constitutively expresses genes in *P. pastoris* cells grown on glucose, glycerol, methanol, and oleic acid [22]. The constitutive GAP promoter based expression vector may be a choice for heterologous protein expression in *P. pastoris* [4–6].

Angiostatin, a tumor associated angiogenesis inhibitor [14, 17], has been cloned and expressed in *E. coli* [12] and inducibly expressed in *P. pastoris* using methanol inducible alcohol oxidase I promoter (pAOX1) [11, 23, 24]. In our previous study, we reported the constitutive expression of human angiostatin in *P. pastoris* using the GAP promoter in shake flask culture and demonstrated that angiostatin secreted from GAP promoter linked system is higher than that from AOX1 promoter based inducible system in shake flask cultures and these two products have the same antiangiogenic and antitumor activity [25]. Since the hazard and cost associated with storage and delivery of large volumes of methanol are eliminated in GAP promoter controlled constitutive expression system and the GAP promoter vectors allow for continuous production of the recombinant product avoiding the fed-batch fermentation using the methanol inducible system, this system is more suitable for large-scale production of heterologous recombinant proteins [1]. In this study we describe the GAP promoter (pGAP) controlled constitutive expression of angiostatin gene in *P. pastoris* by high-density cell culture which is still a new field on gene engineering industrialization to evaluate whether the pGAP can be an alternative to pAOX1 for large-scale production of heterologous proteins in *P. pastoris* by high-density cell culture.

Materials and methods

Cell lines and culture

B16 melanoma cells and C57BL/6J mice were provided by Lab. of Cell Culture and Experimental Animal Center of Sun Yat-Sen University. The cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml streptomycin.

Construction of expression vector pGAP9K-AS and preparation of recombinant *Pichia pastoris* strains

The pGAP is amplified by PCR from *P. pastoris* chromosome DNA. The 5′ primer was 5′-GCAGCGAGC TGATCCTTTTTTGTAT-3′ and the 3′ primer was 5′-GGCCGGATCCTGTGTTTTGATAGTTGTTC-3′. The amplified pGAP was cloned at *Sac*I and *Bam*HI sites of pPIC9K to replace the inducible promoter pAOX1 resulting in the constitutive expression vector, pGAP9K. The human angiostatin gene amplified from the cDNA library [12] was cloned into the *Eco*RI and *Not*I sites of pGAP9K to generate the pGAP9K-AS (Fig. 1). The *Bgl*II linearized pGAP9K-AS was transformed into the *P. pastoris* strain of GS115, which was designated as GS115 (pGAP9K-AS). High angiostatin-producing strains were selected by replicating the transformants on G418 (Neomycin, Invitrogen Corp, San Diego, CA, USA) containing plates. Angiostatin expression was detected by SDS-PAGE.

Fermentation condition and metabolite determination for constitutive expression

Eight 500-ml flasks with 100 ml YPD medium each were inoculated from a frozen GS115 (pGAP9K-AS) stock and incubated at 30°C and 250 rpm for 20 h and transfer to a 20 l fermentor (B. Brown) with a 10 l working volume. A modified growth medium recommended by Invitrogen Corp [8] was used. It consisted of (l) 26.7 ml of 85% H_3PO_4, 9.3 g of $CaSO_4{\cdot}2H_2O$, 18.2 g of K_2SO_4, 14.9 g of $MgSO_4{\cdot}7H2O$, 4.13 g of KOH, 40 g of glycerol, 40 ml of PTM4, 20 g of peptone and 10 g of yeast extracts. The composition of PTM4 trace elements was described as mentioned above [8]. The fermentation was carried out at 30°C and 100–750 rpm by continuously feeding with 50% glycerol-0.8% PTM4. The dissolved oxygen (DO) level was maintained between 25–30% by adjusting the agitation and aerating rate and feeding. pH was controlled at 5 by adding 7 MNH_4OH. Samples were taken every 6 h to measure the biomass and angiostatin, during the course of fermentation.

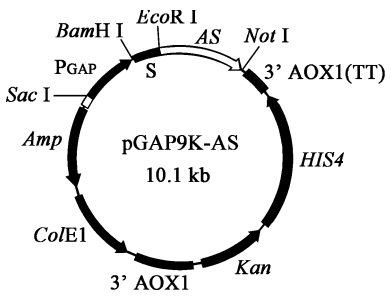

Fig. 1 Physical map of recombinant plasmid. pGAP9K-AS

Angiostatin expression and purification

Samples were taken every 6 h and centrifuged at 5,000*g* for 10 min. The supernatant was used for SDS-PAGE and Western Blot to detect angiostatin expression. For large-scale recovery, the biomass was removed from fermentation broth by centrifugation. The supernatant was loaded on a SP-Sepharose Fast Flow column (Pharmacia Biotech. NJ, USA). The column was then washed with equilibrium buffer (50 mM Na_2HPO_4–24 mM $C_6H_8O_7$). Bound protein was eluted successively with 0.3, 0.6 and 1 mM NaCl. Angiostatin containing fractions were collected, concentrated and desalted with ultrafiltration device (Millipore, Bedford, MA, USA). The concentrated sample was then loaded onto Sephadex G-100 (Pharmacia Biotech, NJ, USA) gel filtration column. Angiostatin was washed down with 0.01 M PBS pH7.2.

Chicken chorioallantoic membrane angio-genesis assay

The anti-angiogenesis activity of the expressed products was determined on 6-day-old chicken embryo chorioallantoic membrane (CAM) by placing a filter paper disc(0.8 × 0.5 cm) on the CAM as described previously [7]. Thirty nanograms of bFGF (basic fibroblast growth factor, Invitrogen) and 25 μg of angiostatin were added on the sterilized filter paper. For control, angiostatin was replaced by PBS. The embryos were incubated at 37°C for 48 h and the filter disc was removed and surrounding CAM tissues were fixed, excised, and photographed. These experiments were performed three times with five embryos each group per condition.

Treatment of B16 melanoma in C57BL/6J mice

The procedure was performed as described in reference [15]. 2.74×10^6 B16 melanoma cells in o.18 ml PBS were subcutaneously injected into 7-week-old C57BL/6J male mice. When the tumors reached 100–200 mm^3 in size the mice were randomly divided into three groups ($n = 4$). Group B was received recombinant angiostatin generated from constitutive expression at a dosage of 100 mg/kg/d. Group A treated with PBS served as control. After 12 days, the mice were sacrificed and tumors excised and weighed. The tumor growth inhibition was calculated according to the formula: Tumor inhibition rate = (average tumor weight of the control group – average tumor weight of the experiment group) × 100%/average tumor weight of the control group [9]. The tumor volume was calculated using the standard formula ($width^2 \times length \times 0.52$). Tumor length and width were measured using a Vernier calipers [14].

Results

Angiostatin production

Unlike pAOX1 which was inactivated on glucose and glycerol and needs methanol to initiate gene expression, pGAP constitutively expresses genes in *P. pastoris* cells grown on glucose, glycerol, methanol, and oleic acid. Before the fermentation for angiostatin production, carbon source and the angiostatin expression was investigated and glycerol appears to be a better carbon source than glucose and methanol in angiostatin secretion (Fig. 2). The fermentation was a fed-batch process. The GS115 (pGAP9K-AS) cells were initially grown on glycerol and then fed-batch with 50% glycerol – 0.8% PTM4 for 60 h. During the fermentation process the culture volume increased from 10.8 to 12.7 l. *Pichia* cell concentration (A_{600}), angiostatin concentration (mg/l), feeding glycerol (ml/h) and DO (%) are described in Fig. 3. As shown in Fig. 3, the biomass accumulated was 305 at A_{600} and angiostatin expression reaches its peak with a yield of 176 mg/l. Figure 4 shows the expression and Western blot analysis of purified angiostatin from *P. pastoris*.

Effect of recombinant angiostatin on in vivo angiogenesis

To test the ability of recombinant human angiostatin to inhibit bFGF induced in vivo angiogenesis, the chick CAM assay was used. As shown in Fig. 5, CAM treated with bFGF and PBS were vascularized (Fig. 5a), whereas CAM treated with bFGF and angiostatin produced by the constitutive (Fig. 5b) or inducible

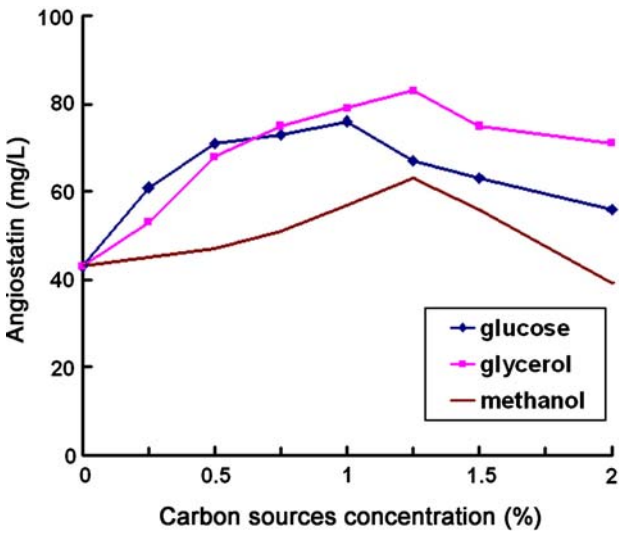

Fig. 2 Carbon sources and angiostatin expression

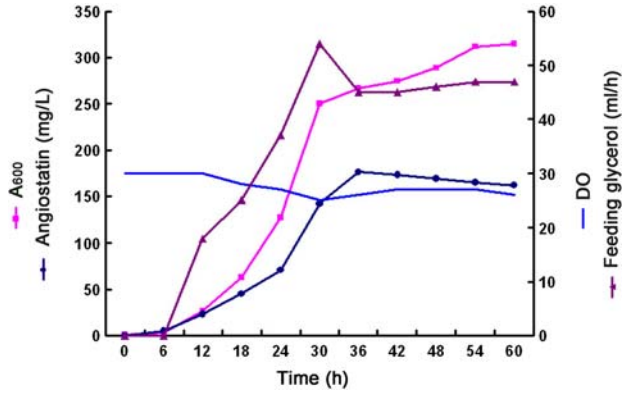

Fig. 3 Accumulation of biomass and angiostatin, feeding glycerol and DO during the high-density cell culture of GS115 (pGAP9K-AS)

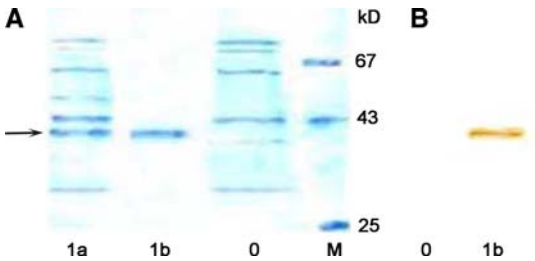

Fig. 4 SDS-PAGE and Western blot analysis of expressed and purified products. **a** SDS-PAGE; **b** Western blot. *0* GS115 (pGAP9K); *1a* GS115(pGAP9K-AS); *1b* Purified protein from *1a*; *M* Protein marker

(Fig. 5c) expression generated discontinuous blood vessels or avascular zones within the filter disk-surrounding region. The results demonstrate that recombinant angiostatin produced from both constitutive and inducible expression significantly inhibit bFGF-induced angiogenesis on chick embryo CAM.

Effect of angiostatin on the growth of tumor in mouse xenograft model

For in vivo experiment, established xenograft tumor model in mice was used to test the effectiveness of angiostatin derived from constitutive expression in *P. pastoris* as an inhibitor of angiogenesis-dependent tumor growth. We examined the effect of purified angiostatin on primary mouse tumor model in C57BL/6J mice. Recombinant angiostatin administered daily at 100 mg/kg, significantly inhibited the growth of B16 murine melanoma by 90.63%, as measured by mean tumor weight (3.224 ± 0.523 g of control group and 0.302 ± 0.052 g of angiostatin group) (Fig. 6b). Tumor volumes changed gradually during the treatment time and on the 12th day after treatment, the tumor volume in the control animals was 3,375 mm^3, whereas in the treated with angiostatin group, it was about 42 mm^3 ($P < 0.05$) (Fig. 6a). Furthermore, in the studies, mice appeared healthy with no signs of wasting, and none of the mice died during treatment.

Discussion

Pichia pastoris has been widely used as an efficient expression system for the production of heterologous proteins, and over several hundred proteins and peptides have been expressed using the pAOX1 linked inducible system of *P. pastoris*. However, some efforts have been made to circumvent the use of methanol and pGAP controlled constitutive system was reported to be a suitable choice and has been used to express several heterologous proteins [1]. In our previous study, we reported the construction of the pGAP based constitutive vector pGAP9K and the constitutive expression of human angiostatin in *P. pastoris* GS115 in shake flask culture using the GAP promoter for the first time and demonstrated that GAP promoter is more efficient than AOX1 promoter for angiostatin expression and reduction of downtime associated with fermentation turnaround [25]. To evaluate the feasibility for large-scale constitutive expression of angiostatin using GAP promoter in *P. pastoris*, a high-density cell culture method to constitutively express human angiostatin in *P. pastoris*

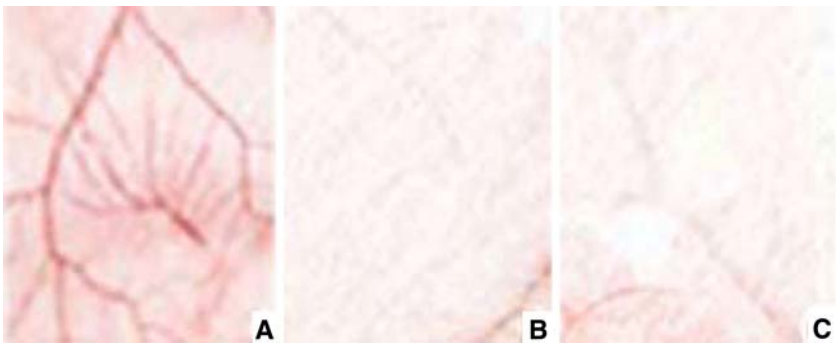

Fig. 5 Constitutive expressed angiostatin inhibits CAM angiogenesis induced by bFGF. **a** bFGF + PBS; **b** bFGF + angiostatin (constitutive expression); **c** bFGF + angiostatin (inducible expression)

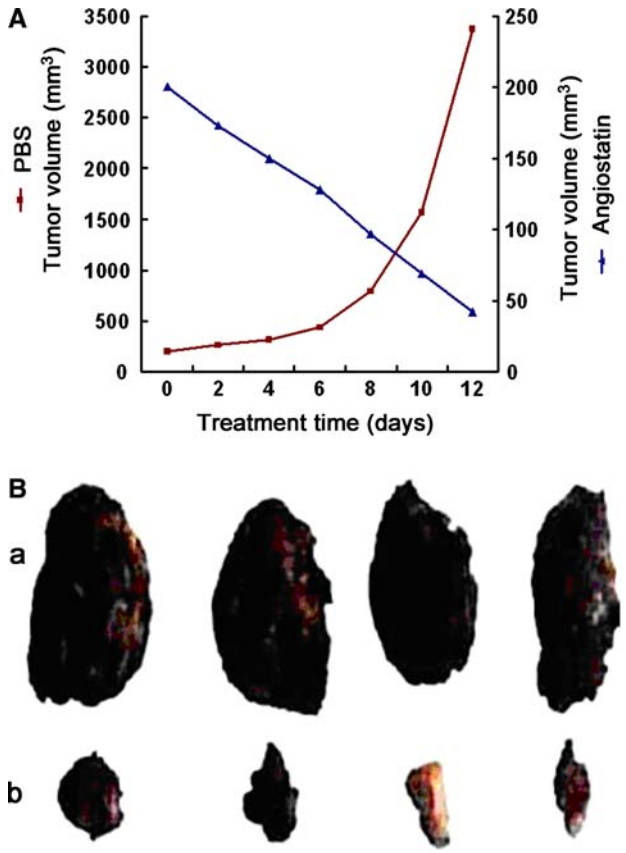

Fig. 6 Effect of recombinant angiostatin on the growth of mouse B16 melanoma. **A** Tumor volume change following angiostatin or PBS treatment; **B** Tumors removed from the mice. *a* Tumors from PBS treated mice. *b* Tumors from angiostatin (100 mg/kg) treated mice

was established in this study. The fermentation was carried out in a 20 l bioreactor with a 10 l working volume and lasted for 60 h but after 36 h of fermentation, the peak angiostatin produced was 176 mg/l, which is two-fold higher than the yield we obtained (58 mg/l) in shake flask culture (25) and also higher than the result from our methanol induced system cultivated in 20 l fermentor with 10 l of working volume for 90 h including 60 h of methanol induction, which produced 135 mg/l of angiostatin (data to be published soon).This expression level is also much higher than the angiostatin produced from pAOX1 based inducible system by Dr. Shiloach's group who established a pilot-scale method to inducibly express human angiostatin in *P. pastoris* in a 15 l fermentor with a 10 l working volume. The fermentation lasted 90 h, of which 70 h were grown on methanol and the culture increase from 7 to 11.5 l during the methanol induction, producing 29 mg/l or 200 mg in total angiostatin [11]. These results suggest that pGAP controlled constitutive system is more efficient in expression level or fermentation time than pAOX1 based inducible system for angiostatin expression in *P. pastoris* by shake flask culture as well as by high-density cell fermentation and GAP promoter is likely to serve as an alternative to AOX1 promoter in large-scale production of angiostatin and other heterologous recombinant proteins.

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