Expression, Purification and Characterization of Recombinant Human Angiogenin in Pichia pastoris

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The potential of angiogenin (Ang) for clinical use has been highlighted in view of its important roles in inducing angiogenesis, facilitating cell proliferation, and inhibiting cell apoptosis. To produce soluble, correctly folded recombinant protein with a high yield, a DNA fragment encoding human Ang was inserted into eukaryotic expression vector pPIC9 and transformed into Pichia pastoris. The expression of recombinant human Ang (rhAng) accounted for about 70% of total secreted proteins. Purifying the Ang from the culture supernatant yielded 30 mg/L at 90% purity by chromatography with a SP Sepharose FF column. Biological assays indicated that rhAng can induce new blood-vessel formation, promote HeLa cell proliferation, increase Erk1/2 phosphorylation, and upregulate c-myc expression. Preparation of bioactive rhAng might lay the basis for further functional study, and might provide an effective strategy for large-scale production of soluble human Ang.

Key words: angiogenin; pichia pastoris; secretory expression; purification; cell proliferation

Angiogenin (Ang) is isolated from serum-free supernatants of an established human adenocarcinoma cell line (HT-29), but is not a tumor-specific product. Expression of Ang is also found in normal cells and normal human plasma. Ang has been found to play roles in tumorigenesis and angiogenesis. In one study, there was a delay in tumor occurrence and progression was slower in Ang antisense-transfected cells. Recent research indicates that Ang also promotes, cell proliferation and survival and inhibits cell apoptosis in addition to angiogenesis. Under stress conditions, it modulates the production of tRNA-derived stress-induced RNAs (tiRNAs), which reprogram protein translation, save anabolic energy, and promote cell survival. It also inhibited serum withdrawal-induced apoptosis in pluripotent P19 mouse embryonal carcinoma cells. With more and more functions of Ang found, more interest is focused on the high-level expression and preparation of Ang to investigate the underlying mechanisms of Ang in many diseases.

Although Ang can be produced in prokaryotic expression systems, the target protein forms inclusion bodies and makes for the subsequent purification process involving a complicated refolding step with a low recovery rate. In addition, there is a report of Ang expression in cultured baby hamster kidney cells, which is not only costly but also unsuitable for commercial large-scale production of therapeutic proteins.

The Pichia pastoris expression system has been used successfully in the production of various recombinant heterologous proteins. Most importantly, P. pastoris is a eukaryote, and hence has the potential to produce soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications required for functionality. In the present study, a human Ang DNA fragment was inserted into eukaryotic expression vector and Ang was purified from the culture supernatant. This recombinant human Ang (rhAng) was bioactive and activated the Erk signaling pathway. We established a more efficient method of producing soluble, bioactive rhAng using a eukaryotic expression system of Pichia pastoris. This lays a basis for further study of its role in possible action mechanisms as well as in clinical applications.

Materials and Methods

Construction of the expression vector. The DNA fragment (378bp) coding for human Ang was released from pUCmT/Ang digested with EcoRI/NcoI and subcloned into vector pPIC9 (Invitrogen, Carlsbad, USA). pUCmT/Ang was constructed previously for Escherichia coli expression in our laboratory. Recombinant plasmid pPIC9-Ang was confirmed by restriction analysis and sequencing.

Transformation and selection of the rhAng expression strain. Pichia pastoris GS115 was transformed with a linearized expression vector digested with BglII. Transformation was performed by the electroporation method as described in the P. pastoris expression manual (Invitrogen). The transformants were analyzed by colony PCR to verify integration of the recombinant gene. Positive transformants were tested for their ability to secrete Ang into the cell-culture supernatants. The cells were inoculated into 5 mL of buffered minimal glycerol (BMGY) medium and were allowed to grow for 36 h, then 1% (v/v) methanol was added to the culture at intervals of 24 h during an incubation period of 120 h. The supernatants were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue G-250 for visualization.

Expression and purification of rhAng. Positive transformants were picked from the plates and inoculated into a flask containing 10 mL of BMGY medium. After shaking at 30 °C at 300 rpm for 24 h, the culture was centrifuged at 4 °C at 3000 rpm for 5 min. The cells collected were

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Abbreviation: rhAng, recombinant human angiogenin
Recombinant Human Angiogenin Production in *Pichia pastoris*

Resuspended in 2 mL of BMMY medium and allowed to grow at 29 °C for 6 d, and then methanol was added every 24 h. To obtain the highest yield of protein, various culture parameters, including temperature, a different vector pHL-L1, other *P. pastoris* SMD1180 cells, and the shaking velocity were tested. Scale-up expression was performed under optimized cultivation conditions. The culture was centrifuged at 5,000 rpm for 15 min and the supernatant was concentrated by ultrafiltration. The concentrated sample was loaded onto an SP Sepharose FF column (Pharmacia Biotech, Uppsala, Sweden) and eluted with a linear gradient of 0–2.0 M NaCl in PBS. The eluted fractions were analyzed by 15% SDS–PAGE.

**Western blot analysis.** Protein samples were separated by 15% SDS–PAGE and electroblotted onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, USA). It was blocked with 5% (w/v) BSA in 10 mmol/L Tris–HCl with 150 mM NaCl (pH 8.0) and 0.1% (v/v) Tween-20 (TBST) for 2 h at room temperature (RT). It was then incubated for 2 h at RT with anti-human Ang monoclonal antibody (R&D, Minneapolis, MN, USA). After washing of it 3 times, it was incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG (ZhongShan, Beijing, China). It was then washed with TBST, and it was incubated for 2 h at room temperature (RT). After washing of it 3 times.

**Chick chorioallantoic membrane assay (CAM).** Fertilized white leghorn chicken eggs (purchased from the Institute of Animal Sciences, Chinese Academy of Agricultural Science) were incubated at a humidity of 70% at 37 °C. On the third day of incubation, an irregular window (0.5 x 1.5 cm) was made at the top of the air chamber at the large blunt end of the eggs. The window was sealed with tape and the eggs were returned to the incubator. On day 10 of development, small plastic rings made with Thermanox™ discs were placed on the CAM (chorioallantoic membrane), and 10–20 μL of various samples was deposited into the rings after gentle laceration of the CAM surface. CAMs were examined daily until day 17, and were collected for microscopy and photographic documentation. Each experimental group included eight eggs, and all experiments were repeated 3 times.

**MTT assay.** The biological activity of rhAng and its commercial product (R&D) were assayed using HeLa cells. The cells were seeded at 5,000 cells/well in a 96-well plate, and various concentrations of rhAng diluted with DMEM containing 10% (v/v) FBS were added to each well, and incubated for 48 h at 37 °C. 5% CO2. Cell proliferation was analyzed by MTT (3-(4,5-dimethylthiazolyl-2)-2,-diphenyltetrazoliumbromide) proliferation assay. The absorbance at 450 nm was measured using a Bio-Rad Model 550 microplate reader (BioRad Molecular Bioscience Group, Hercules, USA).

**Preparation of protein lysates.** HeLa cells were cultured for 24 h, washed with DMEM 3 times, and serum-starved for another 24 h. The cells were then incubated with various concentrations of rhAng for 2 h or with 1 μg/mL of rhAng for various lengths of time. The cells were lysed in lysis buffer. Samples with equal amounts of protein were subjected to SDS–PAGE and Western blotting analyses for phosphorylation of Erk1/2 (Cell Signaling Technology, Boston, USA), c-myc (Cell Signaling Technology) and Ang (R&D Systems).

**Results**

**Construction of the recombinant expression plasmid**

A DNA fragment encoding Ang was inserted between the EcoRI and NotI sites of *P. pastoris* expression vector pPIC9, which contains a *Saccharomyces cerevisiae* alpha factor leader and an alcohol oxidase1 (AOX1) promoter (Fig. 1). Sequencing of the recombinant plasmid pPIC9-Ang confirmed that the reading frame of Ang was correct.

**Transformation and expression of rhAng**

Recombinant vector pPIC9-Ang was transformed into *P. pastoris* GS115 cells by electroporation. The transformed strains were analyzed by colony PCR to evaluate the integration of Ang into the *P. pastoris* transformants. A 378-bp insert was detected in the positively transformed GS115 cells. The positive colonies were cultured and induced to express the recombinant proteins. SDS–PAGE indicated that rhAng was successfully expressed in the *P. pastoris* GS115 transformant. The culture supernatant was harvested from the culture every 24 h after methanol induction. As shown in Fig. 2A, a band corresponding to rhAng with an expected molecular weight of about 14.4 kDa was observed 48 h after induction, and the maximal expression level was reached at 72 h (Fig. 2A). This result was confirmed by Western blot (Fig. 2B). Optimal conditions leading to a maximum Ang yield were as follows: a temperature of 29 °C, a shaking speed of 290 rpm, and an induction time of 72 h. The various vector and *P. pastoris* cells had no effect on IL-22 production.
These results suggest that rhAng could stimulate HeLa cell proliferation by the Erk signaling pathway and the expression of c-myc.

**Discussion**

Ang is the first known human tumor-derived protein with *in vivo* angiogenic activity. It is the only member with angiogenesis ability in the ribonuclease superfamily, and is a unique protein with low ribonucleolytic activity in angiogenesis factors. Some studies have suggested that Ang is involved in tumorgenesis, cell growth, and cell apoptosis and survival. The promise of Ang in clinical applications has been highlighted, particularly for clinical diagnostic criteria of diseases such as cervical cancer and breast cancer.

Ang is produced mainly in prokaryotic expression systems and purified from inclusion bodies. However, this process involves a complicated refolding step for the inclusion bodies. Moreover, the contamination of pyrogenic components derived from *E. coli* is a significant concern in clinical applications. The eukaryotic expression system of *P. pastoris* has become increasingly popular for its ease of genetic manipulation and ability to grow to a high cell density, which makes possible high yields. In addition, pyrogenic contamination is not a concern. Many pharmaceutical proteins have been produced successfully using this system.13)

To produce large amounts of bioactive human Ang for both basic and clinical studies, we tested *P. pastoris* as an expression system. After optimization of expression conditions, Ang accounted for about 70% of total secretory proteins. After purification by SP Sepharose FF column, 30 mg of rhAng was obtained from 1 L of culture supernatant, higher than the Ang yield of COS7 cells in our laboratory (unpublished data). Ang has been produced only as a protein fused with GST in prokaryotic expression systems in our research (unpublished data). An *in vitro* activity assay confirmed that the biological activity of the purified rhAng was comparable to a commercially available product.

Ang also participates in signaling pathways. It activates Erk1/2, induces the transient phosphorylation of protein kinase B/Akt in human umbilical vein endothelial cells, and induces the phosphorylation of SAPK/JNK in human umbilical artery smooth muscle cells.14–16) It induces nitric oxide synthesis in endothelial cells through PI-3 and Akt kinases.17) It also inhibits serum withdrawal-induced apoptosis by activating the NF-xB-mediated cellular survival pathway and the Bcl-2-mediated anti-apoptotic pathway in pluripotent P19 mouse embryonal carcinoma cells.6,7) In our study, rhAng stimulated Erk signaling pathway in a dose-dependent manner and in a short time in HeLa cells, and upregulated the expression of oncogene c-myc. The results suggest that rhAng stimulates HeLa cell proliferation by the Erk signaling pathway and the expression of c-myc. Hence preparation of bioactive rhAng in the eukaryotic expression system is valuable for clarifying the biological effect of Ang on cell growth.

In conclusion, we have developed a novel, highly efficient way of producing biologically active rhAng, one that can be adopted for large-scale production of functional rhAng. This work should help to promote...
studies of the clinical application of Ang and of the mechanisms of action for Ang.

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Fig. 4. The Angiogenesis Effects of rhAng on the Chick Embryo Chorioallantoic Membrane.
A, Stimulatory effect of rhAng on angiogenesis in the chorioallantoic membrane. Lane 1, culture supernatants P. pastoris transformed with pPIC9 as negative control; lane 2, commercially available Ang as positive control; lane 3, culture supernatants from P. pastoris transformed with pPIC9-Ang. B, Number of capillary vessels. Level 1, number of blood vessels around the sample-inoculated regions; level 2, number of blood vessels intersecting with the fifth ring. Lanes 1–3, as in Fig. 4A.

Fig. 5. Activity Assay of rhAng.
A, The indicated amounts of rhAng and a commercial product were added individually to 96-well plates containing 5,000 cells/well. After incubation for 48 h, cell growth was measured by MTT assay. Data represent the means for three independent experiments. B, The expression of phosphorylated Erk1/2, c-myc, and Ang in Ang-treated HeLa cells. The cells were incubated with 0, 10, 50, 250, and 1,000 ng/mL Ang for 2 h. C, Expression of phosphorylated Erk1/2 and Ang in Ang-treated HeLa cells. The cells were incubated with 1,000 ng/mL Ang for 0, 5, 10, 30, 60, and 120 min.

References


