Full Paper

Functional expression of LZ-8, a fungal immunomodulatory protein from *Ganoderma lucidum* in *Pichia pastoris*

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LZ-8, a fungal immunomodulatory protein found in *Ganoderma lucidum*, has been proposed to have therapeutic effects on cancer and autoimmune diseases. To develop an efficient and facile expression system supplying of rLZ-8 of high purity and stable activity for further study and applications, a full-length cDNA of *lz8* gene was cloned into the pPIC9K to construct a yeast expression vector pPIC9K-lz8, then transformed to *Pichia pastoris* strain GS115. The molecular weight of the rLZ-8 was about 17 kDa. The yield of rLZ-8 reached up to 270 mg L⁻¹ at optimal culture conditions. In vitro, the rLZ-8 was capable of hemagglutinating mouse red blood cells, but no such activity was observed toward human red blood cells, which were similar to the native LZ-8. The mouse splenocyte proliferation activity of rLZ-8 expressed in *P. pastoris* was much higher than the rLZ-8 expressed in *E. coli*. All these results suggested that the active rLZ-8 protein can be expressed efficiently in the *P. pastoris* expression system. This report may supply a new economic and convenient way for the application of LZ-8 protein.

Key Words—express; immunomodulatory activity; LZ-8 protein; *Pichia pastoris*

Introduction

The fruiting bodies of *Ganoderma lucidum* are a well known Chinese crude drug component which has been used clinically in China, Japan and Korea for a long time as a home remedy. It is considered able to promote longevity and maintain the vitality of humans (Lieu et al., 1992). Thus, many studies have focused on isolating the pharmaceutically active components to understand the underlying mechanisms (Lin and Zhang, 2004; Ma et al., 2003), and have showed that glycoprotein is an important substance with various activities. Fungal immunomodulatory proteins (FIPs)

were an important active glycoprotein for their immunomodulation function. Until now, four FIPs, termed LZ-8 (Kino et al., 1989), FIP-fve (Ko et al., 1995), FIP-vvo (Hsu et al., 1997) and FIP-gts (Lin et al., 1997), were purified from *G. lucidum* and other species of mushroom.

As the first FIP found in mushroom, the structure and biological functions of LZ-8 have been explored extensively. LZ-8 shows considerable similarity to the variable region of the immunoglobulin heavy chain in both its amino acid sequence and predicted secondary structure (Tanaka et al., 1989). As for anti-tumor activity, LZ-8 has been found to be a mitogen in vitro for human peripheral blood lymphocytes, mediated by activating macrophages and T lymphocytes to induce the production of cytokine and tumor necrosis factor- α (Haak-Frendscho et al., 1993; Vander Hem LG et al., 1995). In addition, LZ-8 has also been proved to modify the expression of adhesion molecules on immuno-

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competent cells (Miyasaka et al., 1992), to suppress autoimmune diabetes in young female non-obese diabetic mice (Kino et al., 1990), and to prevent antibody production and systemic anaphylaxis (Kino et al., 1991). Therefore, LZ-8 might be a good candidate for developing a new type of functional food supplements or therapeutic agents to be used for the prevention or treatment of cancer and autoimmune diseases.

Direct extraction of native LZ-8 from *G. lucidum* is costly and time-consuming. As reported earlier, only 5–10 mg of purified LZ-8 was obtained from 340 g of *G. lucidum* mycelia (Kino et al., 1989). Moreover, the biological activity of the purified LZ-8 is usually dependent on the quality and quantity of *G. lucidum* sources, which limited the further study and application of LZ-8. To develop an efficient and facile expression system supply of LZ-8 of high purity and stable activity for further study and applications is important. In this study, the *P. pastoris* expression system was used to produce a high yield of LZ-8 and its immunoactivity was also investigated.

Materials and Methods

Chemicals, strains and media. DNA restriction enzymes were purchased from the TaKaRa Corporation. A Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology. RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). Concanavalin A (ConA) was obtained from Sigma (St. Louis, USA).

G. lucidum, strain HG, was obtained from the Shanghai Academy of Agricultural Sciences and maintained on potato dextrose agar (PDA). *P. pastoris* strain GS115 (Invitrogen), used for the expression of LZ-8 protein, was grown, transformed and analyzed according to the manufacturer's instructions. The expression vector pPIC9K was purchased from Invitrogen Corporation. *P. pastoris* was grown in buffered minimal glycerol medium (BMGY) and in buffered minimal methanol medium without glycerol, containing 0.5% (v/v) methanol (BMMY) for heterologous expression.

Cloning of *G. lucidum* lz8 gene. Based on the published *G. lucidum* lz8 sequence (GenBank Accession Number: J05735) (Murasugi et al., 1991), two specific primers were synthesized. The nucleotide sequences of these primers were as follows: ATGTCCGACACTGCCTTG (lz8F, forward primer) and GATCGAAT-

TCTAGTTCCACTGGGCGATG (lz8R, reverse primer, *Eco*RI site in bold face).

PCR amplification was carried out in 0.2-ml tubes using a Programmable Thermal Cycler PTC200 (MJ Research, Inc., Watertown, MA, USA). Amplification conditions were 30 cycles at 94°C for 30 s, 56°C for 40 s, and 72°C for 40 s, followed by a final extension at 72°C for 5 min.

Construction of LZ-8 recombinant expression vector and expression of recombinant LZ-8 in *P. pastoris*

The 330-bp PCR product was digested with *Eco*RI and ligated into *Eco*RI and *Sna*BI restriction sites of pPIC9K (Invitrogen) to construct the plasmid pPIC9K-lz8 (Fig. 1). Then the resultant expression construct was used to transform *E. coli* XL1-blue strain. The construct was confirmed by sequencing, linearized with *Bgl*III, and transformed by electroporation in *P. pastoris* strain GS115. The phenotype of the colonies obtained was checked as recommended by the supplier Invitrogen. The His⁺ transformants were placed in microtiter plates until all clones were at the same density. The cultures were then spotted on the YPD-geneticin plates and scored for geneticin resistance. To screen for geneticin resistant transformants, the four YPD plates containing each of the following concentrations of geneticin (0, 0.25, 0.5, 0.75, 1.0, 2.0 mg ml⁻¹) were prepared.

The transformants with phenotype MutS plus a negative control with pPIC9K alone were screened for the expression of recombinant LZ-8. 50 ml BMGY growth media was inoculated with single colonies of *P. pastoris*. The cells were grown at 30°C for 24 h at a shaker speed of 300 rpm. The cells were harvested when the culture reached an optical density of 3–6 at 600 nm

Fig. 1. Schematic diagram of construction of pPIC9K-lz8.

The 336 bp lz8 cDNA was cloned into the pPIC9K plasmid using the *Sna*BI and *Eco*RI restriction sites, and expressed in yeast strain GS115 under the control of the 5' AOX1 promoter.

(OD₆₀₀), and resuspended in 100 ml BMMY induction medium. Methanol-induced cultures were grown at 30°C while being shaken at 300 rpm and continuously induced for 4 days by adding methanol (0.5%, v/v final concentration) every 24 h. After 4 days, the cells were removed by centrifugation and supernatant was analyzed by SDS-PAGE.

SDS-PAGE analysis. For SDS-PAGE, 10 µl supernatant of the *P. pastoris* with pPIC9K-lz8 and pPIC9K (mixed with 10 µl 2× sample buffer respectively) were loaded on the SDS-PAGE gel. All samples were boiled for 5 min and stored at 4°C prior to electrophoresis (Laemmli et al., 1970). SDS-PAGE was conducted in 15–25% (w/v) acrylamide gel and stained with Coomassie brilliant blue G250.

Optimal production of recombinant LZ-8 in the *Pichia pastoris* expression system. *The optimal induction methanol concentration:* The *P. pastoris* cells were cultured at 100 ml BMMY induction medium with different methanol concentrations (v/v) (0.1%, 0.5%, 1%, 2% and 5%) at 28°C. The same amount of methanol was added each day. The fermentation broth was collected after induction for 5 days, the cells were removed by centrifugation and the concentration of rLZ-8 in the supernatant was determined by the Bradford method (Bradford, 1976).

The optimal induction time: To determine the optimal induction time, *P. pastoris* cells were cultured as before, but induced with the optimal induction methanol concentration. Aliquots of expression medium (2 ml) were collected at the time points of 24, 48, 72, 96, 120, 144, and 168 h, and the cells were removed by centrifugation. Then the protein concentration of each supernatant was monitored and the optimal induction time was obtained.

The optimal initial pH value: Different optimal initial pH value (5.8, 6.4, 7.0, 7.6 and 8.0) were also observed when cells grown at the optimal methanol concentration for the optimal induction time. The other culture conditions were the same as before. The protein concentration of supernatant in different fermentation broth was examined as before. All the experiments were performed at least in triplicate.

Hemagglutination assay. Hemagglutination activity was measured as described previously (Stavitsky, 1954). After washing the whole blood with PBS three times, blood cells were collected by centrifugation at 300 × g for 10 min and then suspended to 1.5% (v/v) with PBS. The rLZ-8 expressed in *E. coli* (Ding and

Zhao, 2006) (10 µg ml⁻¹) and in *P. pastoris* (10 µg ml⁻¹) (25 µl) and 1.5% blood (25 µl) were placed in 96-well U-bottom microtiter plates and incubated at room temperature. The plates were examined for hemagglutination after 2 h.

Effect of rLZ-8 on the splenocyte proliferation activity. Four-week-old male or female C57BL/6 mice were sacrificed by cervical dislocation, and the spleens were removed using sterile techniques. The murine splenocytes were collected after passing through a wire screen (100 mesh), and red blood cells in the cell suspension were hemolyzed with 0.16 M NH₄Cl, 0.17 M Tris-HCl (pH 7.7). After washing the cells with 10 ml RPMI 1640 and centrifuging them at 2,000 rpm for 10 min, the cells were resuspended in 10 ml RPMI 1640 medium containing 10% FBS, 100 units ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin (pH 7.3), then seeded onto a 96-well microtiter plate (200 µl well⁻¹). An equal quantity of sample was added to each well. Cell proliferation was estimated by the CCK-8. After incubation of the cells at 37°C under 5% CO₂ in air for 72 h, 10 µl CCK-8 solution was added to each well and incubated for 4 h at 37°C. The absorbance of each well was determined using the microplate reader (Molecular Devices Co., USA) at 450 nm. The cell proliferation ratio = A_{450} of experimental group / A_{450} of negative control × 100%.

Statistical analysis. The results were expressed as the mean ± S.D. of data obtained from triplicate experiments. The statistical significance was estimated using a Student's *t*-test for unpaired observations. Values with *p* < 0.05 were considered significant.

Results

*Heterologous expression of the LZ-8 in *Pichia pastoris**

Agarose gel analysis of the product amplified by primers lz8F and lz8R revealed the presence of a band of approximately 330 bp. The *lz8* gene was cloned into the pPIC9K vector to generate a recombinant LZ-8 construct pPIC9K-lz8. Sequencing of the whole gene confirmed the *lz8* gene in correct frame (Fig. 1). Transformation into *P. pastoris* with linearized vectors resulted in numerous colonies. The transformants with multiple copy inserts were identified by the screening of the high concentration of geneticin, and had the phenotype of His⁺ and MutS.

SDS-PAGE analysis of rLZ-8 produced in *P. pastoris*

After induction, the protein supernatant was resolved on SDS-PAGE gel. Compared with the negative control, an extra band of approximately 17 kDa was found in the supernatant of the *P. pastoris* with pPIC9K-lz8 (Fig. 2, Lane 2). Other proteins were not detected by the SDS-PAGE. As expected, a mass of accumulation of rLZ-8 was detected in the culture medium. This result was nearly identical to the mass of natural LZ-8 protein, and little larger than the theoretical molecular mass calculated by amino acid sequence (Kino et al., 1989; Murasugi et al., 1991).

Optimal fermentation conditions for recombinant LZ-8 in *Pichia pastoris*

The result of the optimal induction methanol concentration is shown in Fig. 3A. At the methanol concentration of 0.5%, the rLZ-8 could be induced abundantly, while from the methanol concentration of 0.5% to 5%, the expression level didn't change significantly. Therefore, the methanol concentration of 0.5% was used in the later experiment.

At the methanol concentration of 0.5%, the optimal induction time was tested and the result is shown in Fig. 3B. The maximum expression level of rLZ-8 occurred after induction for 144 h. During hours 144 to 168, the expression level decreased. Thus, the induction time of 144 h was used in the later experiment.

The optimal pH value was also observed when cells were induced at 0.5% methanol concentration for 144 h. As Fig. 3C shows, from the initial pH value of 5.8

to 8.0, the expression level was increasing. At the initial pH of 8.0, the yield reached up to 273 mg L⁻¹.

Hemagglutination reaction

To test the hemagglutination activity of rLZ-8 expressed in *P. pastoris*, human red blood cells of four types (A, B, AB, and O), and sheep red blood cells were used. The hemagglutination activity of rLZ-8 expressed in *E. coli* was also examined. With mouse red blood cells, aggregation was observed in the presence of rLZ-8 produced in *E. coli* and *P. pastoris* at 10 µg ml⁻¹. But no aggregation reaction was observed toward human red blood cells (A, B, AB, and O types). The hemagglutination activity of rLZ-8 expressed in *E. coli* and *P. pastoris* is similar to the native LZ-8 protein.

Fig. 2. SDS-PAGE analysis of rLZ-8 produced in *P. pastoris*.

Lane M: precision protein standard with molecular weights given in kilo Daltons. Lane 1: the supernatant of the *P. pastoris* with pPIC9K-lz8. Lane 2: the supernatant of the *P. pastoris* with pPIC9K as a negative control.

(A)

(B)

(C)

Fig. 3. The optimal expression conditions for rLZ-8 production in the *Pichia pastoris* expression system.

The optimal condition of rLZ-8 expression was examined in different methanol concentrations (A), time courses (B) and initial pH values (C). These data are shown as mean ± S.D.

For a control, all of the types of human red blood cells or mouse red blood cells became aggregated in the presence of Con A.

Effect of rLZ-8 on the splenocyte proliferation activity

To determine the immunomodulation activity of rLZ-8 expressed in *P. pastoris*, the splenocyte proliferation activity of rLZ-8 expressed in *P. pastoris* was examined using the CCK-8 Kit. The splenocyte proliferation activity of rLZ-8 expressed in *E. coli* was also tested. As Fig. 4 shows, the rLZ-8 expressed in *P. pastoris* had a strong splenocyte proliferation activity at $5 \mu\text{g ml}^{-1}$ compared to the negative control; the proliferation ratios was 149% (PBS control served as 100%). However, there was no significant difference between the activity of rLZ-8 expressed in *E. coli* and the negative control at $5 \mu\text{g ml}^{-1}$.

Discussion

In order to produce sufficient FIPs for further study and application, several studies about the heterologous expression of FIPs were performed. As reported elsewhere, FIP-fve has been expressed in *E. coli*, but the activity was lower than that of the native FIP-fve (Ko et al., 1997). A similar result is also observed when the rFIP-gts is expressed in *E. coli* (Lin et al., 1997). Our result also suggested that activity of rLZ-8 expressed in *E. coli* had no significant splenocyte proliferation activity. The rFIPs expressed in *E. coli* may not be folding properly and without glycosylation, the activity is lower than that of the native FIPs (Jinn et al., 2006). Therefore some reports are tending to express active rFIPs in insect cell systems for medicinal applications and genetic study. The activity of the protein expressed by insect cells is higher than that of the product in *E. coli* (Jinn et al., 2006; Wu et al., 2008). However, the expression level in insect cells is moderate. Wu et al. showed that the yield of the rFip-fve produced was 6.25 mg L^{-1} in the infected Sf21 cell supernatant (Wu et al., 2007), which is much lower than the rLZ-8 produced in *P. pastoris* (Fig. 3). At the same time, the rFIPs produced in insect cells are not suitable for large-scale production. To overcome these shortcomings, the *P. pastoris* expression system was used in this study to express rLZ-8 protein.

In this study, we got a large amount of rLZ-8 protein from the *P. pastoris*, and the product was easy to purify. We also observed that the hemagglutination activity of

Fig. 4. Effect of the rLZ-8 expressed in *E. coli* cells (rLZ-8 *E. coli*) and in *P. pastoris* cells (rLZ-8 *P. pastoris*) on splenocyte proliferation by CCK-8.

Splenocyte treated various materials (PBS, ConA, rLZ-8 *E. coli*, rLZ-8 *P. pastoris*) at $5 \mu\text{g/ml}$ for 72 h. After stimulation, $10 \mu\text{l}$ CCK8 solutions were added to each well and cultured at 37°C for another 4 h. The absorbance of each well was determined at 450 nm. Each column and each bar represents the mean $\pm$ S.D. PBS: negative control; ConA: positive control. * $p < 0.05$, ** $p < 0.01$, compared with the negative control.

rLZ-8 expressed in *P. pastoris* was similar to the native LZ-8 in vitro (Kino et al., 1989), and the splenocyte proliferation activity was much higher than the rLZ-8 expressed in *E. coli* (Fig. 4). At the same time, the expression level of rLZ-8 in *P. pastoris* was much higher than that in insect cells (Fig. 3). Therefore, the active rLZ-8 protein can be expressed efficiently in the *P. pastoris* expression system. Moreover, for *P. pastoris* are lower eukaryota as well as *G. lucidum*, so they may provide a similar environment for the production of proteins. Therefore, next to *E. coli* cells, mammalian and insect cells, *P. pastoris* may be a much suitable choice for the expression of LZ-8 protein. This is the first report using the *P. pastoris* expression system for the heterologous expression of FIPs.

Because the four kinds of FIPs found in mushroom have similar structure and biological activity (Kino et al., 1989; Ko et al., 1995), this report may also provided certain basis for the expression of other FIPs in *P. pastoris* expression systems.

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