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Production of Recombinant Humanized Anti-HBsAg Fab Fragment from *Pichia pastoris* by Fermentation

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In this report, we describe the high-yield secretory expression of the recombinant human anti-HBsAg Fab fragment from *Pichia pastoris* that was achieved by co-integration of the genes encoding the heavy and light chains (both under the control of alcohol oxidase promoter) into the genome of the yeast cells. The fed-batch fermentations were carried out in a 5 L scale. Both chains of the Fab were successfully expressed upon methanol induction. The absorbance (OD_{600}) of the broth can reach 350~500 at the end of fed-batch phase. After the induction, the expression level of the recombinant Fab (soluble) reached 420~458 mg/L. The recombinant Fab fragment was purified from the crude culture supernatant by ion exchange chromatography and the purity of the recombinant Fab fragment was over 95%. The affinity activities of the crude fermentation supernatant and the purified Fab were analyzed by indirect ELISA, which showed that the purified recombinant Fab fragment had high affinity activity with hepatitis B surface antigen.

Keywords: Fab, Fermentation, HBsAg, *Pichia pastoris*

Introduction

Antibodies play a crucial role in protecting the human body against viral infections, and the parenteral administration of antibodies can be an effective therapeutic and preventative measure against measles, polio, hepatitis and other viral infections (Dulbecco and Ginsberg, 1980; White and Fenner, 1986). The patients infected with Hepatitis B virus (HBV) may incur chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Blum *et al*., 1998). Passive immunoglobulin (Ig) using anti-hepatitis B Ig (HBIG) effectively prevents the infection of hepatitis B virus in patients who undergo liver transplantation with hepatitis B surface antigen (HBsAg)-positive allografts (Muller *et al*., 1991) and infants born to HBsAg-positive mothers (Seef, 1984). However, since HBIG is prepared from the sera of anti-HBsAg antibody-positive donors, it is difficult to ensure that the preparation is free of unknown infectious agents. Monoclonal antibodies and their fragments are not only useful in the prevention if they bind to and block surface regions of the virus, but also essential for the infection of hepatocytes.

Fab fragments are particularly important for a wide variety of applications, including detection and treatment of human diseases (Levy and Miller, 1983), *in vitro* diagnostic test (Tam and Goldstein, 1985) and affinity purification methods (Johnstone and Thorpe, 1982). Anti-HBsAg Fab fragment has a considerable potential in the prevention and treatment of liver diseases caused by HBV. However, to date, high-level expression of the anti-HBsAg Fab fragment is still a big challenge. Humanized anti-HBsAg Fab fragment was expressed in *Escherichia coli* at levels of 10 mg/L in periplasm (Maeda *et al*., 1999) and 80mg/L in formation of inclusion body by fermentation (An *et al*., 2003). In our laboratory, the high-yield secretory expression (30~40 mg/L) of the recombinant human anti-HBsAg Fab fragment from *Pichia pastoris* in shake flask was achieved by co-integration of the genes encoding the heavy and light chains (both under the control of alcohol oxidase promoter) into the genome of the yeast cells (Deng *et al*., 2002). In the present experiment, higher cell densities of the recombinant yeast and higher expression of the recombinant humanized anti-HBsAg Fab fragment were achieved by means of fed-batch fermentations.

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Materials and Methods

***Pichia pastoris* strain** The *P. pastoris* strain was achieved by co-integration of the genes encoding the heavy and light chains (both under the control of alcohol oxidase promoter) of the recombinant humanized anti-HBsAg Fab fragment into the genome of the yeast cells, which have mut^s phenotype and can secret anti-HBsAg Fab fragment at a level of about 40 mg/L in shake flask (Deng *et al*., 2002).

Growth media In preparation for the fermentation, two types of media were used in the study. The rich medium BMGY contained 1% yeast extract (OXOID), 2% peptone (OXOID), 10% YNB (made up of 100 g/L $(NH_4)_2SO_4$ and 34 g/L yeast nitrogen base, Invitrogen Co.), 2 mL/L biotin solution and 2%(v/v) glycerol (Sino-American Biotechnology Co.). Basal salt medium contained 85% o-phosphoric acid (26.7 ml/L), 0.93 g/L calcium sulfate·$2H_2O$, 18.2 g/L potassium sulfate, 14.9 g/L magnesium sulfate·$7H_2O$, 4.13 g/L potassium hydroxide, 40 g/L glycerol and 12 mL trace metal salt (PTM_1). The basal salt medium was then filter-sterilized and stored at 4°C. The PTM_1 salt solution contained 6.0 g/L cupric sulfate · $5H_2O$, 0.08 g/L sodium iodide, 3.0 g/L manganese sulfate · H_2O, 0.2 g/L sodium molybdate·$2H_2O$, 0.02 g/L boric acid, 0.5 g/L cobalt chloride, 20.0 g/L zinc chloride, 65.0 g/L ferrous sulfate·$7H_2O$, 5.0 ml sulfuric acid, and 0.2 g/L biotin. Antifoam 289 (Sigma) was used as the foam suppressor. 50% glycerol supplemented with basal salt was used for glycerol feed in the fed-batch phase. 50% methanol supplemented with 12 mL/L PTM_1 was used for methanol feed in the induction phase.

Shake flask culture protocol The shake flask experiments were carried out in 500 mL baffled flasks (50 mL working volume). The minimum glycerol medium (1.34% yeast nitrogen base, 2% glycerol, 0.0004% biotin) was used. The culture was incubated at 30°C with shaking (300 rpm). At an absorbance of 20 (OD_{600}), cells were harvested and re-suspended in 50mL minimum methanol medium (1.34% yeast nitrogen base, 0.0004% biotin). Methanol (final concentration was respectively at 0.5%, 1.0%, 1.5%, 2%, 2.5%, 3%) was added every 24 h during the methanol induction phase. After 96 h induction, the supernatant was collected and the quantitative analysis of recombinant Fab fragment was carried out.

Fed-batch fermentations All fermentations were carried out in a 5-L bioreactor (Biostat B-5, Braun Co., Germany) with control modules for pH, temperature, agitation, dissolved oxygen (DO) and air flow. Seed culture for the fermentation was started from the fresh glycerol stock and inoculated directly into baffled flask (50 mL working volume) containing the minimum glycerol medium. After 24 h of growth, seed culture was inoculated with 1% inoculum. Seed culture was grown for 16~18 h and was used to inoculate for the fermentation. Five percent inoculum was used for inoculation of a 5 L bioreactor containing 2 L of basal salt medium. The aeration rate was initially maintained at 1.0 vvm and then increased to 1.5 vvm when required. Dissolved oxygen was kept above 20% saturation. The pH of the medium was controlled at 5.0 by adding 25% ammonia hydroxide. Temperature was maintained at 30°C.

Fermentation was operated in batch mode until all of the glycerol was consumed as indicated by a sharp rise in dissolved oxygen concentration (approximately 30 h). To achieve high cell density, a glycerol feed (50% glycerol containing 12 mL/L PTM_1) was then maintained for about 10~20 h at a rate of 15mL/L/h (the feeding rates were based on the volume in the bioreactor during the batch phase). A methanol feed (50% methanol containing 12 mL/L PTM_1) was then maintained for as long as 96 h. The feeding rate of methanol (initially ~2.7 mL/L/h) was gradually increased (up to 22.5 mL/L/h) to keep the methanol concentration at 0.5~1.0% and the DO level between 20% and 30%. The methanol concentration of the broth was detected by colorimetry in real time during the methanol feed batch phase.

Biomass analysis Total cell concentration was determined by measuring the absorbance of the broth at 600 nm (OD_{600}) in a spectrophotometer. Samples were diluted if the absorbance was above 0.6. To determine the biomass concentration in terms of cell weight, samples of the culture broth (100 mL) were taken in duplicate, centrifuged at 6000 rpm and washed twice with deionized water. They were dried to constant weight at 90°C, a process which took 48 h. One unit of OD_{600} was found to be equivalent to ~ 0.45 mg dry cell weight.

SDS-PAGE and Western blot analysis 10 μL of fermentation supernatant was mixed with 10 μL of 2×SDS-sample buffer (5% SDS, 10% glycerol, 0.002% bromophenol blue, 0.0062 M Tris-HCl, pH 7.0), supplemented with 2% mercaptoethanol for gels run under reduced conditions, and separated on 12.5% polyacrylamide gels with a 4% stacking gel (Laemmli, 1970). For protein estimations, Coomassie-stained SDS-gels were analyzed by densitometry using Scion Image.

The proteins separated by SDS-PAGE were electro-transferred to polyvinylidene difluoride (PVDF) membrane (Milipore, Massachasett, USA) by the method of Sambrook (Sambrook *et al*., 1989) (Bio-Rad transblot SD; 15V, 30 min). The membrane was incubated with HRP (horseradish peroxidase)-conjugated goat anti-Human Fab-specific IgG (Sigma, St. Louis, USA, 1:1000 dilution in 1% albumin/PBS-buffer) for 1 h at 37°C. The reactive bands were visualized by OPD (Promega, Madison, USA) staining.

Purification of the recombinant Fab The recombinant Fab fragment in the cultural supernatant was precipitated by 40% $(NH_4)_2SO_4$. The sediment was dissolved in 0.02 M Tris buffer (pH 8.0) and desalted with Sephadex-G-25 column. The recombinant Fab fragment was purified by affinity chromatography (HiTrap chromatography column conjugated with goat anti-human Fab IgG; Pharmacia, Uppsala, Sweden).

Affinity activity assay Each well of a microtiter plate (Sino-American Biotechnology Co.) was coated with 50 μL of Na-carbonate buffer (pH 9.6) containing 200 ng/ml HBsAg (Zhongshan Bioengineering Co.). After coating overnight at 4°C, the wells were washed five times with 0.05% Tween-20/PBS (PBS-T) and non-specific binding sites were blocked with 200 μL of 2% BSA/PBS per well for 2 h at room temperature. The culture supernatant, purified Fab fragment (100 μg/mL) and HBIG (10 IU/mL) (produced by the Research Institute of Biological Products in Shanghai, China; used as a positive control) were serially diluted

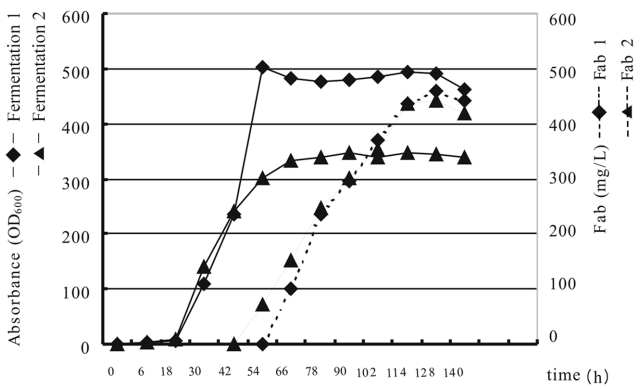

Fig. 1. Curves for cell density and expression of recombinant Fab fragment in fermentation.

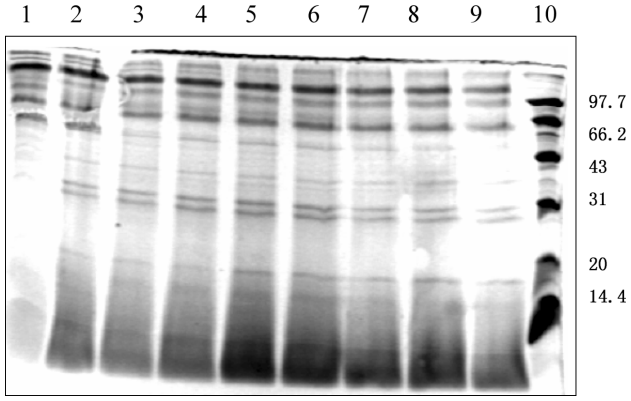

Fig. 2. SDS-PAGE analysis of culture supernatant at different times. Lane 1, culture supernatant before the induction; Lanes 2~9, culture supernatant after the induction by methanol at different times; Lane 10, standard molecular weight marker.

and added to the wells and the incubation continued for 2 h at 37°C. After five washings with PBS-T, 50 μL of HRP conjugated goat anti-human Fab IgG (Sigma Co.) was added at a dilution of 1/1000 in PBS and the incubation continued for 1 h at 37°C. After five washings with PBS-T, 50 μL of O-phenylenediamine substrate solution (Sigma, St. Louis, USA) was added and incubated for 10 min at 37°C. The reaction was stopped by the addition of 50 μL of 2 N H_2SO_4. The absorbance of each well was measured at 450 and 630 nm with an ELISA reader (Denley Dragon MK2, Finland).

Results

Strategy of induction For increased productivity of the recombinant protein in *Pichia pastoris*, it is desirable to achieve high cell density by methanol. In our experience, it is not that the higher cell density before the induction, the higher target protein expressed. The culture absorbance (OD_{600}) reached a value of 501 before the induction, the concentration of the recombinant Fab fragment reached a value of 458mg/L after 72 h and 443 mg/L after 96 h in the methanol induction phase. The culture absorbance (OD_{600}) reached a value of 241 before the induction, the concentration of the recombinant Fab fragment could reach 420 mg/L after 96 h in the methanol induction phase (Fig. 1). The optimum culture absorbance (OD_{600}) before the induction should be 250~350. If the cell density were too high (OD_{600} is over a value of 400) before the induction, the fermentation process would be difficult to control, because too much foam would be produced in the fermentation broth, and too much antifoam had to be added, which would make it difficult to precipitate the target protein from the fermentation supernatant by $(NH_4)_2SO_4$. Furthermore, the dissolved oxygen is the main limitation factor in fermentation, the high cell density (OD_{600} is over a value of 400) would result in the shortage of dissolved oxygen.

High methanol concentration in the fermentation broth is toxic to cells. The effect of addition of various concentration of methanol during the methanol induction was evaluated in shake flask experiments (Table 1). The best concentration of methanol was 10 g/L. The addition of higher amount of methanol to the culture resulted in a decrease in the expression level of Fab fragment. During the methanol induction phase, a slow feeding rate of methanol was initiated so that the cells could gradually adapt to the methanol environment. After 3-4 h of growth on methanol, the feeding rate was increased linearly from 1 to 3 ml/L/h at a 24 h interval. When the cells adapted to the methanol environment, the feeding rate of methanol should be adjusted to keep its concentration at 10 g/L in the fermentation broth.

SDS-PAGE and Western blot analysis The SDS-PAGE analysis of samples was shown in Fig. 2, Two bands at approximately 28 kD in reduced condition and one band at approximately 50 kD in non-reduced condition were verified

Table 1. Effect of addition of various concentration of methanol in shake flask

Methanol concentration (g/L)	Initial OD_{600}	End OD_{600}	Initial pH	End pH	Fab concentration (mg/L)
5	9.6	29	6.0	6.3	37.4
10	9.6	29.1	6.0	6.2	40.48
15	9.6	30.4	6.0	6.5	35.8
20	9.6	28.6	6.0	6.7	31.8
25	9.6	23.7	6.0	6.9	20.6
30	9.6	18.2	6.0	7.1	14.6

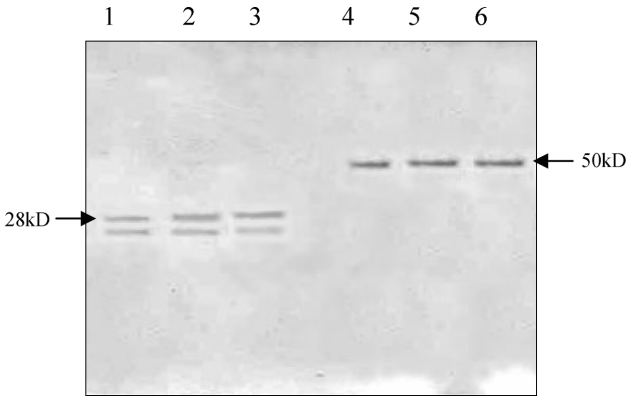

Fig. 3. The western blot analysis of the recombinant Fab fragment. Lanes 1~3; recombinant Fab in reduced condition, Lanes 4~6, recombinant Fab antibody in non-reduced condition.

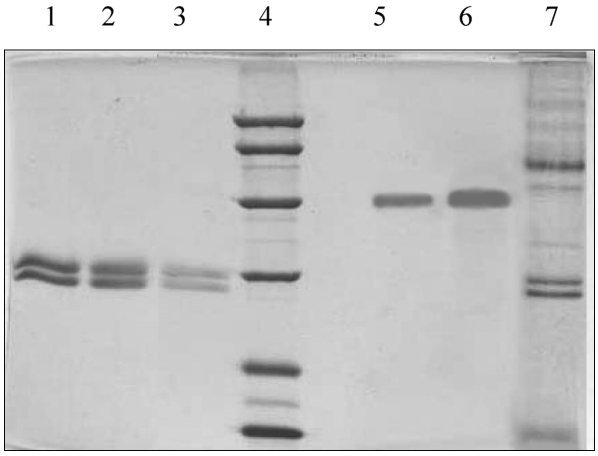

Fig. 4. SDS-PAGE analysis of the recombinant Fab fragment purified from the culture supernatant. Lanes 1~3, purified Fab in reduced condition; Lane 4, standard molecular weight marker; Lanes 5~6, purified Fab in non-reduced condition; Lane 7, culture supernatant.

to be recombinant Fab fragments with a western blot analysis (Fig. 3) using goat anti-human Fab specific IgG.

Purification of the recombinant Fab fragment The recombinant Fab fragment was purified by affinity chromatography. The purified recombinant Fab fragment was analyzed by SDS-PAGE (Fig. 4). Coomassie-stained SDS-gels were analyzed by densitometry using Scion Image. The purity of the purified Fab fragment is over 95%. 14mg purified Fab fragment was obtained from 50 mL culture supernatant. The recovery rate of Fab fragment was above 75%.

Affinity activities The fermentation supernatant, the purified Fab fragment and humanized HBIG led to the expected sigmoid curve (Fig. 5), whereas the negative control (supernatant of induced *P. pastoris* containing pPICZa-A)

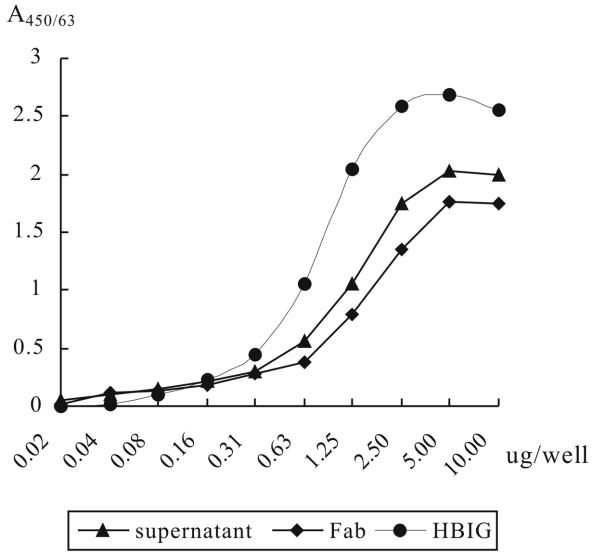

Fig. 5. Quantification of the recombinant humanized anti-HBsAg Fab fragment by ELISA. Serially diluted solutions of the purified recombinant Fab fragment (▲) and serially diluted culture supernatant (◆) in microtiter plates precoated with 200 ng/well HBsAg, Serially diluted solution of standard HBIG (●) was used as a positive control.

gave no signal. The affinity activities of the purified anti-HBsAg Fab fragment were quantified by comparing their values of $A_{450/630}$ with those of serially diluted solutions of standard HBIG preparation, which is currently used at a dose of 200 international units (IU)/time, for passive immunoprophylaxis against HBV. The molecular weight of the Fab fragment was one-third that of the monoclonal antibody (mAb) and these antibodies had one and two antigen-binding sites respectively. Although it is difficult to directly determine the activities of mAb and Fab fragment, taking the differences in their sizes and numbers of antigen-binding sites into consideration, the amount of the antibodies produced could be estimated by their absorbances of direct ELISA. 1mg of recombinant Fab fragment was equivalent to 40 IU HBIG.

Discussion

The methylotrophic yeast, *Pichia pastoris* has proved to be an outstanding host for high level production of both secreted and intracellular proteins (Tschopp *et al*., 1987; Brierley *et al.*, 1990; Clare *et al*., 1991). This organism has the potential for high level expression, efficient secretion (Clare, 1991) and growth to very high cell densities (Digan *et al*., 1989). The success of the Pichia system is linked to the strong tightly regulated alcohol oxidase (AOX1) promoter (Tschopp *et al*., 1987). The strong promoter, coupled with the high cell density fermentation, has allowed the production of recombinant products at high levels (Cregg *et al*., 1987).

The expression level of the recombinant Fab fragment is a

big challenge for clinical application. Heterologous expression in *Pichia pastoris* has many advantages as eukaryotic expression, such as proper folding and disulfide bond formation, glycosylation and secretion. Compared with other eukaryotic systems, *P. pastoris* yeast can be grown on minimal nutrients and can be used to express large quantities of proteins that are not easily obtained from *E. coli*, and adapted for high cell density fermentation (Cregg *et al*., 1993; Chauhan *et al*., 1999; Files *et al*., 2001), which makes this system attractive for the production of heterologous proteins. In our prior study, it was shown that humanized anti-HBsAg Fab fragment can be high level expressed in *P. pastoris*, and the glycosylation has little effect on the affinity activity of the recombinant Fab (Deng *et al*., 2003).

In the present study, the recombinant humanized anti-HBsAg Fab fragment was high-level expressed in *Pichia pastoris* using a fed-batch fermentation system. The quantity of the recombinant Fab fragment was up to 420~458 mg/L in 5 L bioreactor, which is much higher than that of other Fab fragments expressed in *P. pastoris* (Takahashi *et al*., 2000; Lange *et al*., 2001). The recombinant humanized anti-HBsAg Fab fragment was purified effectively by ion-exchange chromatography from the culture supernatant. The affinity activity assay showed that 1mg of the recombinant humanized anti-HBsAg Fab fragment was equivalent to 40 IU HBIG.

In conclusion, the recombinant humanized anti-HBsAg Fab fragment was high-level expressed in *P. pastoris* using a fed-batch fermentation system. The optimum initial absorbance (OD_{600}) of recombinant yeast during the methanol induction phase should be 250~350, the concentration of methanol should be 10 g/L. The results suggested that *P. pastoris* is a powerful organism for high-level expression of the recombinant antibody fragments. The recombinant Fab fragment produced in this study has a strong potential for future clinical application.

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