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Expression, Purification, and Characterization of Humanized Anti-HBs Fab Fragment

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Anti-HBs Fab fragment has considerable potential for use in the prevention and treatment of liver diseases by HBV. Here we established a high-level expression system to directly produce anti-HBs Fab fragment in *Pichia pastoris*. This was achieved by co-integration of the genes encoding the heavy and light chains both under the genome of the yeast cells. The Fab fragment was efficiently secreted into medium at a concentration of 50 mg/liter. The authenticity of the Fab fragment was confirmed by immunoblot analysis, which yielded one band of ~50 kDa under nonreducing conditions and two bands of ~28 kDa under reducing conditions. The anti-HBs Fab fragment was prepared with a purity of 95% by affinity chromatography. The affinity activity of the recombinant Fab was detected by ELISA, which indicated that 1 mg of recombinant Fab was equivalent to 40 IU HBIG (20 IU/mg). The results demonstrated that the recombinant Fab fragment could sufficiently neutralize the HBsAg.

Key words: Fab, expression, purification, *Pichia pastoris*.

The development of phage display technology (*1–3*) and ribosome display technology (*4*, *5*) has given rise to many humanized antibodies and triggered the further development of antibody engineering. Fab fragments are particularly important for a wide variety of applications, such as detection and treatment of human disease (*6*), in vitro diagnostic test (*7*) and affinity purification methods. Hepatitis B infection is a public health problem worldwide. Patients infected with Hepatitis B Virus (HBV) may progress to chronic liver disease including chronic active hepatitis, cirrhosis, and hepatocellular carcinoma (*8*). The anti-HBsAg antibody can neutralize the virus and protect the liver by blocking HBV attachment. So we can use the antibody as a passive immunization agent to protect the infants born to mothers who are hepatitis B surface antigen (HBsAg) positive (*9*) and patients who need liver transplantation for HBV-related liver disease (*10*). The humanized anti-HBs Fab fragment has the same affinity and specificity to antigen as the full-length anti-HBsAg antibody (IgG), and glycosylation has no effect on the affinity of Fab antiobdy (*11*).

In our laboratory we have obtained the genes of anti-HBs Fab from human peripheral blood lymphocytes of a volunteer with high antibody titer to HBV by using of phage display technology (*12*). The anti-HBs Fab gene has been expressed in *E. coli* (*13*), and the recognition of epitopes by the Fab fragment has been demonstrated (*14*). This showed that the anti-HBs Fab fragment has high affinity and specificity to HBsAg antigen, but the expression level of Fab fragment in *E. coli* was low.

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The expression level of recombinant Fab fragment is the main factor limiting its industrial application. 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, *P. pastoris* can be grown on minimal nutrients and adapted for high cell density fermentation (*15*, *16*), which makes this system attractive for production of heterologous proteins. The expression of atrazine-specific Fab and anti-human high affinity IgE receptor Fab by using of *P. pastoris* system has been described (*19*, *20*). In this paper, we report the establishment of a high level expression system to directly produce recombinant anti-HBs Fab in *P. pastoris*, and the purification and specificity analysis of the recombinant Fab fragment.

MATERIALS AND METHODS

Top10′F (Sino-American Biotech Co.) was used as a host for plasmids. GS115 strain of *P. pastoris* (Invitrogen Co.) was used as a host for yeast transformation. The vector pPICZα-A (Invitrogen Co.) was used for construction of yeast expression vectors. Plasmid pCom3H, which contains the gene of L chain and Fd chain of anti-HBs Fab, was used as the model of polymerase chain reaction (PCR). The primers 5′-CGGAATTCCAGGTGCAGCTGGTGGAGTCT-3′ and 5′-GGGGTACCTTAGCTAGTTTTGTCACAAGA-3′ were used for amplification of the Fd chain of Fab. The primers 5′-GGAATTGTGTTGACCCAGTCT-3′ and 5′-CGGAATTCCTAACACTCT CCCCTGTTGA-3′ were used for the amplification of the L chain of Fab. The L chain and Fd chain were verified by sequencing and inserted into the windows of pPICZα-A digested with *Eco*RI and *Xba*I, respectively, to construct vectors pPICZα-

A-H and pPICZα-A-L. Fd chain expression cassette digested by *Bgl*II and *Bam*HI from pPICZα-A-H was inserted into the window of pPICZα-A-L digested by *Bam*HI to construct expression vector pPICZα-A-H-L.

Construction and Selection of Recombinant Yeast GS115/Fab—Expression vector plasmid pPICZα-A-H-L linearized with *Sac*I was introduced into GS115 cells by electroporation (1.50 kV, 200 Ω). The transformants grew well in YPD (1% yeast extract, 2% tryptone, 2% dextrose) plates with 100 μg/ml Zeocin™. The transformants were transferred into YPD plates with 500, 1,000, and 1,500 μg/ml Zeocin™ to select the transformants in which multiple copies of expression cassettes were inserted into the genome. The transformants were detected by PCR using the primer specific for the genes of Fd chain and L chain of the Fab.

Production of Recombinant Fab Fragment—The selected transformants was cultured in 50 ml of BMGY (100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 1% glycerol, 0.00004% biotin) at 37°C for 24 h. The cells were collected and resuspended in 100 ml of BMMY (100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 0.5% methanol, 0.00004% biotin) and cultured for an additional 96 h at 30°C with vigorous shaking to induce the anti-HBs Fab fragments.

SDS-PAGE and Western Blotting Analysis—The cultural supernatant of recombinant yeast GS115/Fab was treated with non-reducing buffer (5% SDS, 10% glycerol, 0.002% Bromophenol blue, 0.0062 M Tris-HCl, pH 7.0) or reducing buffer (5% SDS, 10% glycerol, 0.002% Bromophenol blue, 0.0062 M Tris-HCl, pH 7.0, 2% mercaptoethanol), and proteins were separated with 12% SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) by the method of Laemmli (*17*).

The proteins separated by SDS-PAGE were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Milipore Co.) by the method of Sambrook (*18*). The membrane was incubated with HRP (horseradish peroxidase)-conjugated goat anti-Human Fab-specific IgG (Sigma Co.) for 1 h at 37°C. The reactive bands were visualized by NBT/BCIP (Promega Co.) staining.

Purification and Character Analysis of Recombinant Fab Fragment—The cultural supernatant of recombinant yeast GS115/Fab was precipitated with 40% $(NH_4)_2SO_4$. The sediment was dissolved in 0.01 M Tris-HCl (pH 8.0) and desalted with Sephadex-G-25 column. The desalted sample was loaded on HiTrap affinity chromatography column (Pharmacia Bio Co.), equilibrated with 0.1 M and 0.01 M Tris-HCl buffer (pH 8.0) and then eluted by 0.1 M Glycerol buffer (pH 3.0).

The analysis of glycosylation of Fab was carried out by MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), performed at the Measurement Center of Zhongshan University. The purified Fab fragment was treated with endoglycosidase H (Sigma Co.). A 0.2 mg portion of Fab fragment was dissolved in 400 μl of 100 mM sodium acetate buffer (pH 5.5), incubated for 12 h at 37°C with 0.01 U of endoglycosidase H, desalted and freeze-dried, and its molecular mass was determined by MALDI-TOF-MS.

Enzyme Linked Immunosorbent Assay (ELISA)—Each well of a microtiter plate (Sino-American Biotech Co.) was coated with 50 μl of Na-carbonate buffer (pH 8.0) containing 200 ng/ml HBsAg (Zhongshan Bioengineering Co.) overnight at 4°C. The wells were washed five times with 0.05% Tween-20/PBS (PBS-T), then non-specific binding sites were blocked with 100 μl of 2% BSA/PBS per well for 2 h at room temperature. The purified Fab fragment (100 υg/ml) and HBIG (10 IU/ml, produced by the Research Institute of Biological Products, Shanghai, China) were serially diluted and added to the wells, then incubated for 2 h at 37°C. After washing three times with PBS-T, the wells were filled with 50 μl of HRP-conjugated goat anti-human Fab-specific IgG (Sigma Co.) diluted with 1/10,000 in PBS and incubated for 1 h at 37°C. The wells were filled with 50 μl of *O*-phenylene- diamine substrate solution (Sigma Co.) and incubated for 5 min at 37°C, then 50 μl 2 M of H_2SO_4 was added to stop the reaction. The absorbance was measured at 450/630 nm with an ELISA reader (Denley Dragon MK2, Finland).

RESULTS

Expression of Recombinant Fab—The pPICZα-A-H-L expression vector, which contains the sequences encoding both Fd and L chains of the anti-HBs Fab fragment, each fused directly in frame with the the α-factor signal sequence under the control of the AOX1 promoter, was constructed in two steps (Fig. 1). The Fd and L chains of anti-HBs Fab were individually fused in frame with the α-factor signal sequence of pPICZα-A vector and

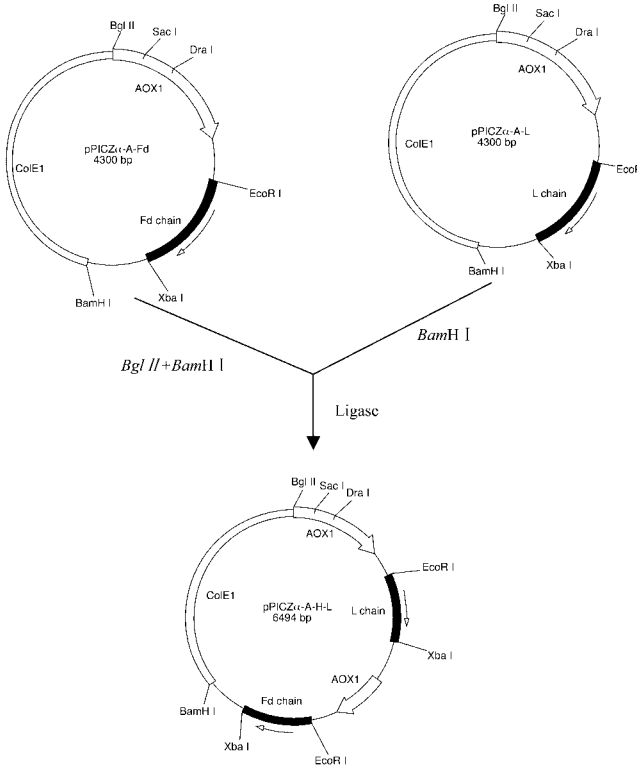

Fig. 1. **Construction of expression vector pPICZα-A-H-L.** The genes of light chain and Fd chain were each subcloned into pPICZα-A vector. The expression cassette of the Fd chain digested with *Bgl*II and *Bam*HI was inserted into pPICZα-A-L to construct expression vector pPICZα-A-H-L.

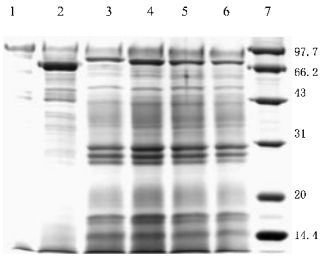

Fig. 2. **SDS-PAGE analysis of supernatant of recombinant yeast GS115/Fab.** Lane 1, supernatant of yeast GS115/ pPICZa-A as negative control; lane 2, supernatant of recombinant yeast GS115/HSA as positive control; lanes 3–6, supernatant of recombinant yeasts GS115/Fab under reducing conditions; lane 7, molecular weight marker.

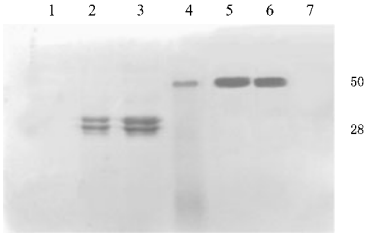

Fig. 3. **Western-blot analysis of supernatant of recombinant yeast GS115/Fab.** Lanes 1 and 7, supernatant of yeast GS115/ pPICZa-A as negative control; lanes 2 and 3, supernatant of recombinant yeast GS115/Fab under reducing conditions; lane 4, molecular weight marker; lanes 5 and 6, supernatant of recombinant yeast GS115/Fab under non-reducing conditions.

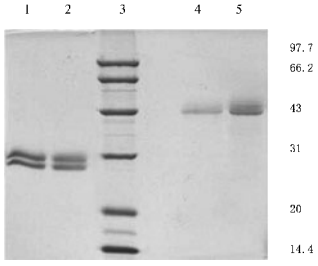

Fig. 4. **SDS-PAGE analysis of Fab fragment purified by affinity chromatography.** Lanes 1 and 2, purified Fab fragment under reducing conditions; lane 3, standard molecular weight marker; lane 4 and 5, purified Fab fragment under non-reducing conditions.

sequenced. The resulting expression vector was linearized with *Bgl*II and introduced into GS115 cells. The transformants in which the AOX1 gene was replaced by the expression cassettes as the result of homologous recombination were selected on Zeocin™ plates. The sequence encoding the Fd and L chains of Fab was integrated into the genome of GS115 to produce recombinant yeast GS115/Fab, and its presence was confirmed by PCR using genomic DNA of transformants as template and antibody gene-specific primers. The selected transformants were grown in BMGY medium and transferred into BMMY medium to induce the gene expression of Fab. The cultural supernatant of recombinant yeast was analyzed by SDS-PAGE (Fig. 2), and two bands of recombinant Fab fragment were observed with molecular mass of approxi-

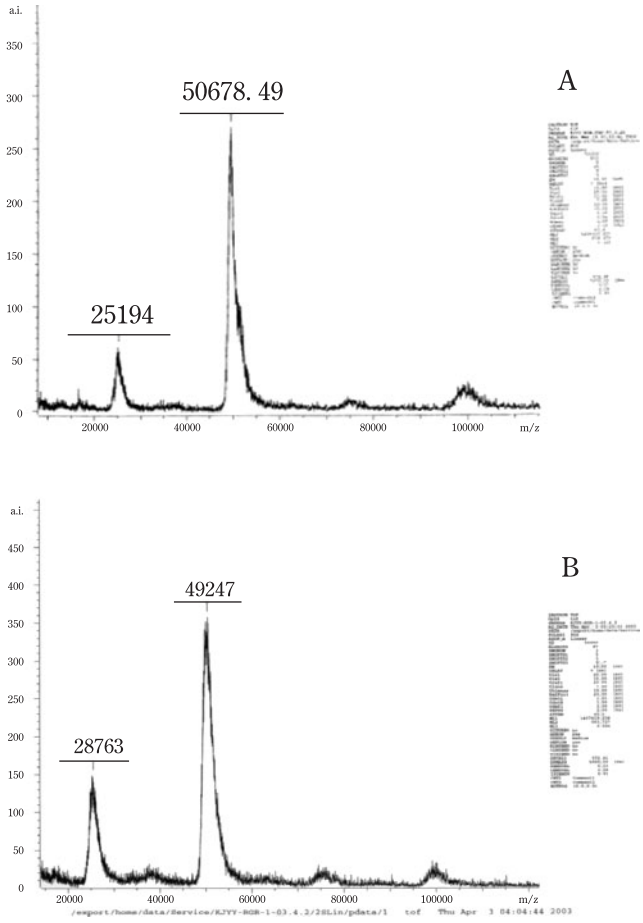

Fig. 5. **Molecular mass of recombinant Fab determined by MALDI-TOF-MS.** A: Molecular mass of the purified recombinant Fab. B: Molecular mass of the purified recombinant Fab treated with endoglycosidase H.

mately 28 kDa. Western blotting revealed one reactive band of recombinant Fab fragment with molecular mass of approximately 50 kDa under nonreducing conditions and two reactive bands of recombinant Fab fragment with molecular mass of approximately 28 kDa under reducing conditions (Fig. 3).

The secreted recombinant Fab fragment was purified from the cultural supernatant with an anti-Fab antibody conjugated HiTrap column to a purity of over 95%. The purified recombinant Fab fragment was analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 4).

Glycosylation Analysis of the Recombinant Fab Fragment—The recombinant Fab fragment was treated with endoglycosidase H, which acts on *N*-linked oligosaccharides/proteins and cleaves high mannose structures. The molecular mass of recombinant Fab fragment digested with endoglycosidase H was determined by MALDI-TOF-MS to be 49247 Da (Fig. 5). This is lower than the molecular mass of normal recombinant Fab fragment detected by MALDI-TOF-MS (50,678.49 Da) by 1,431.49 Da, equivalent about 8 mannose groups. And it is still higher than the theoretical value (47,906.65 Da). It is hypothe-

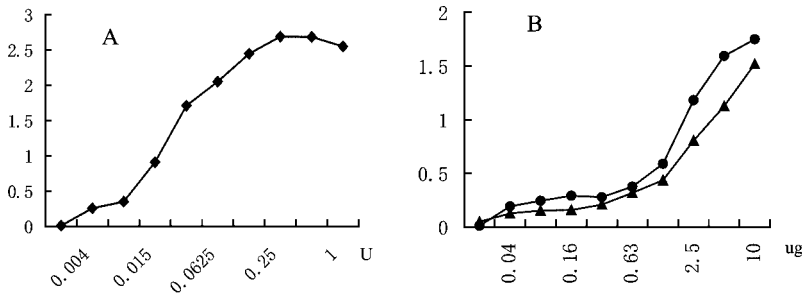

Fig. 6. **Quantification of anti-HBs Fab fragment by ELISA.** A. Serially diluted solution of standard HBIG (solid diamonds) was used as a positive control. B. Serially diluted solutions of purified recombinant Fab fragment (solid circles) and serially diluted supernatant of induced recombinant yeast GS115/Fab (solid triangles) in microtiter plates precoated with 200 ng/well HBsAg.

sized that o-linked oligosaccharides may be present in the recombinant Fab fragment.

Binding Properties of Recombinant Fab Fragment—The affinity activities of recombinant Fab were determined by ELISA. The purified Fab fragment, the cultural supernatant and humanized HBIG gave the expected sigmoidal curves (Fig. 6), whereas the negative control (supernatant of *P. pastoris* transformed with vacant vector pPICZa-A) gave no signal. The activities of the purified anti-HBsAg Fab fragment were quantified by comparing their $OD_{450/630}$ values with those of serially diluted solutions of standard HBIG preparation, which is a currently used at a dose of 200 international units (IU)/time, for passive immunoprophylaxis against HBV. The molecular mass of the Fab fragment was one-third that of the monoclonal antibody (Mab), and these antibodies had respectively one and two antigen-binding sites. Although it is difficut to directly determine the activities of Mab and Fab, taking the differences in their sizes and numbers of antigen-binding sites into consideration, it was estimated from their absorbances on indirect ELISA that 1 mg of recombinant Fab fragment was equivalent to 40 IU.

DISCUSSION

Antibodies have diagnostic and therapeutic value in addition to research use. They are often used in the form of Fab antibodies, which have the advantages of low immunogenicity, rapid accumulation at the targeted tissue and ease of addition of drugs.

Here we showed for the first time that recombinant *P. pastoris* cells secrete a functional anti-HBs Fab fragment after induction with methanol. A single expression vector encoding the L and Fd chains of Fab in separate expression cassettes but with the same regulatory sequences in both AOX1 promoters and a termination signal was constructed. Thus, the L and Fd chains of Fab are both integrated into the genome of transformants.

The expression level (50 mg/liter) of anti-HBs Fab fragment is a little higher then that of other Fab fragments expressed in *P. pastoris* (*19*, *20*). The Fab fragment was secreted into the medium and easily purified without cell disruption and refolding.

The affinity activity of humanized recombinant anti-HBs Fab fragment produced in *P. pastoris* was identical with that of human derived HBIG. According to our ELISA results, 1 mg of anti-HBs Fab fragment was equivalent to 40 IU HBIG (Fig. 6). For clinical applications, antibodies with high affinities and high specificities are required. In this respect, the recombinant Fab fragment appears to have enough affinity, neutralizing activities and specificity to the HBsAg for the clinical application.

Yeast and mammalian cells recognize the same peptide signal [Asn-Xaa-(Ser/Thr)] for glycosylation and utilize a similar pathway for core oligosaccharide synthesis in the endoplasmic reticulum (*21*). The type and extent of outer chain glycosylation appear to be quite different in these cell types (*22*). The molecular mass of the recombinant anti-HBsAg Fab fragment was higher than the theoretical value and decreased when the fragment was digested with endoglycosidase H. This could be attributed to glycosylation, since the Fab fragment has one N-glycosylation site. The results showed that the anti-HBs Fab fragment is a glycoprotein, and that glycosylation has little effect on its activities.

SDS-PAGE showed that the molecular mass of the Fab fragment under reducing and nonreducing conditions is different. This is probably due to intramolecular disulfide bonds, which are intact under nonreducing conditions but broken under reducing conditions. The molecular mass of the Fab estimated by SDS-PAGE and measured by MALDI-TOF-MS is much different from the theoretical value. This is probably due to the glycosylation. The presence of oligosaccharide chains may affect the mobility of glycoprotein in SDS-PAGE.

In conclusion, we showed that humanized anti-HBsAg Fab could be expressed in high level by *P. pastoris*, and that glycosylation has little effect on the affinity activity. *P. pastoris* is particularly well suited for fermentative growth, and has the ability to reach very high cell densities during fermentation and improve overall protein yields (*23*, 24). Therefore, the possibility of anti-HBs Fab fragment production for use in diagnostic or therapeutic applications could be fulfilled by *P. pastoris*.

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