African Journal of Biotechnology Vol. 10(60), pp. 12840-12846, 5 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.190
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Construction of a fusion gene containing hepatitis B virus *L* gene and *Mycobacterium tuberculosis Ag85B* gene and its expression in *Pichia pastoris*

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Accepted 11 August, 2011

This study aimed at exploring the method of secretory expression of hepatitis B virus *L* gene and *Mycobacterium tuberculosis Ag85B* fusion gene in *Pichia pastoris* GS115, and preliminary tests of L-Ag85B fusion protein immunoreactivity. The hepatitis B Virus genome extracted from HBsAg positive serum and genome of *M. tuberculosis* H37Rv was used as template to amplify *L* and *Ag85B* gene. *L-Ag85B* fusion gene was cloned into vector pPICZαC and the expression plasmid pPICZαC-L-Ag85B was constructed. After linearization, the plasmid was transformed into *P. pastoris* GS115 by electroporation. SDS-PAGE and Western blot were used to analyze the expression of protein. The purified fusion protein L-Ag85B was respectively assayed with serum containing anti-HBsAg or anti-Ag85B by ELISA. The recombinant vector was confirmed by sequencing and enzyme digestion. The results of SDS-PAGE and Western blot showed that the recombinant protein was induced by methanol and stably expressed in *P. pastoris*, while it has specific reaction with the serum containing anti-HbsAg or anti-Ag85B. However, the successful construction of a recombinant yeast expression vector containing gene coding L protein and Ag85B gene, and expressed in P. pastoris, lays a foundation for further researches on immunogenicity and immune protection by the new type of Hepatitis B- Tuberculosis combined vaccine.

Key words: Hepatitis B virus, mycobacterium tuberculosis, L gene, Ag85B gene*, Pichia pastoris*, combined vaccine.

INTRODUCTION

Hepatitis B (HB), caused by hepatitis B virus (HBV), and tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB), are very serious infectious diseases. One-thirds of the world's 6.0 billion population have been infected with HBV or MTB (Parida and Kaufmann, 2010; Poorolajal et al., 2010). The HBV vaccine applied at present is primarily composed of HBsAg (S protein) expressed by Chinese hamster ovary cells (CHO cells) and beer yeast. It has been found in vaccination that there are still 10 to 20% of the population who are unresponsive or weakly responsive to the HBV vaccine, or who even do not produce antibodies at all (Kubba et al., 2003; Jin et al., 2006). Bacillus Calmette–Guerin (BCG), a vaccine used to prevent TB, has been widely used in neonatal vaccination for nearly 100 years, yet its protection rate is only about 50% (Kaufmann et al., 2010). The gastro-intestinal mucosa and broken skin are also an important route of transmitting tuberculosis besides the respiratory tract. Along with migration and HIV infection, some new, easily-spreading TB strains and multiple drug resistant strains deteriorate the efficacy of traditional anti-TB drugs (Kaufmann et al., 2010; Okada and Kita, 2010). It is very likely that HBV and TB may become endemic in the immunized population and cause new public problems. Therefore, the development of a novel vaccine superior to the yeast HBsAg and BCG vaccines is urgently needed.

In this study, we constructed vector pPICZαC-L-Ag85B for the expression of a fusion between the HBV *L* antigen and the MTB *Ag85B* gene. We successfully expressed the fusion construct in *P. pastoris* strain GS115, in order

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to further study the immune response and immune protection of the HB and MTB combined vaccine.

MATERIALS AND METHODS

The following materials, *MTB H37Rv* and *E. coli* Top 10, were obtained from the Department of Microbiology and Immunology of the Medical College of Jinan University, while *P. pastoris* GS115 and pPICZαC were obtained from Invitrogen, Shanghai, China. HbsAg positive serum (serum of hepatitis B patients), anti-HbsAg (Hepatitis B surface antigen) positive serum, that is, serum of convalescent hepatitis B patients and anti-Ag85B positive serum (serum of MTB patients), were obtained from the First Affiliated Hospital of Jinan University. The *M. tuberculosis* genomic DNA extraction kit was from Shanghai DOBIO Company (Shanghai, China). *Ex Taq DNA* polymerase, *T4* DNA ligase, restriction enzymes KpnI, EcoRI, HindIII and SacI, DNA marker, Plasmid mini Kit and Gel Extraction Kit were obtained from TAKARA (Dalian, China). The target genes were sequenced by Sangon Biological Engineering Technology and Service Co. Ltd (Shanghai, China). Mouse anti-human HBsAg monoclonal antibody was purchased from Beijing Biosynthesis Biotechnology (Beijing, China) and rabbit anti-Ag85B monoclonal antibody was from Abcam Company (Shanghai, China). However, Ni-Agarose Resin for 6x His-Tagged Proteins were from CWBIO (Beijing, China).

PCR amplification of the HBV L and MTB Ag85B genes

Two pairs of primers were designed based on the sequence of HBV *L* (GI: 157091234) and MTB *Ag85B* (GI: 6469794). HBV *L* gene sense primer P1, 5'- TAT*GAATTC*A*ATG*GGAGGTTGGTCTT CCAAA-3', which contains an EcoRI site and the translation initiation codon; and anti-sense primer P2, 5'-GGCGGAAGCTTAATGTATACCCAAAGAC-3', which primes at the 3'-terminus of the HBV L gene, but omits the translation termination codon. P2 contains a HindIII site. Hepatitis B DNA was extracted from a hepatitis B surface antigen positive serum by using phenol / chloroform extraction methods, and the DNA was used as the template to amplify HBV *L*. The PCR reaction was carried out as follows: 95℃ for 10 min; 95℃ for 50 s; 56℃ for 50 s; 72℃ for 1 min with a total of 35 cycles; and an additional extension step of 10 min at 72℃ which was added at the end.

MTB *Ag85B* gene P3 (sense primer): 5'-TAT*AAGCTTGGATCCGGAGGTTCA*TTCTCCCGGCCGGGGCTG-3' includes a HindIII site and a linker sequence, while P4 (anti-sense primer): 5'-AAT*GGTACC*ATGCCGGCGCCTAACGAAC-3' includes a KpnI site and omits the translation termination codon. The whole genome was extracted from MTB *H37Rv* by a *M. tuberculosis* genomic DNA extraction kit (DOBIO, Shanghai, China), and was used as a template to amplify MTB *Ag85B*. The reaction conditions were at 94℃ for 5 min, 94℃ for 45 s, 52℃ for 45 s and 72℃ for 60 s, after 30 cycles of amplification, and finally at 72℃ for 10 min. The HBV *L* and MTB *Ag85B* PCR fragments were purified by gel extraction.

Construction of the pPICZαC-L-Ag85B plasmid

The purified HBV *L* and MTB *Ag85B* PCR products were digested with EcoRI / HindIII and KpnI / HindIII, respectively. Both fragments were then fused and inserted into the vector pPICZαC which had been cut with EcoRI and KpnI, by ligation with T4 DNA ligase. The resulting expression plasmid was named as pPICZαC-L-Ag85B. The plasmid was transformed into freshly prepared calcium chloride competent *E. coli* TOP10 cells, and the transformed cells were spread on the LLB (1% Low Salt LB and tryptone; 0.5% yeast extract; 0.5% Nacl; PH 7.0) plates containing 25 μg/mL Zeocin. The recombinant plasmid pPICZαC-L-Ag85B was isolated from the selected positive transformants and confirmed by PCR, restriction analysis and sequencing.

Yeast transformation and selection

Competent *P. pastoris* strain GS115 was prepared. The plasmids pPICZαC-L-Ag85B was linearized with SacI, and then transformed into competent *P. pastoris* GS115 by electroporation according to the *P. pastoris* transformation instruction manual (Invitrogen, USA). Positive transformants were selected on YPDS medium (Yeast extract peptone dextrose medium, with yeast extract 1%; peptone 2%; dextrose 2%; and sorbitol 1 M) containing 100 μg/mL zeocin. The Mut+-phenotype was confirmed for both yeasts on MDH (Minimal Dextrose Medium+Histidine, YNB 1.34%; biotin 0.004%; dextrose 2%; histidine 0.004%) (YNB, 1.34% Yeast Nitrogen Base with Ammonium Sulfate without Amino Acids, biotin 0.002%; histidine 0.04%; dextrose 2%; methanol 0.5%; glycerol 1%; 0.1 M potassium phosphate buffer, PH 6.0) and MMH (Minimal Menthanol Medium+ Histidine, YNB 1.34%; biotin 0.004%; menthanol 0.5%; histidine 0.004%) plates following the manufacturer's instructions.

Identification of recombinant yeast by PCR

Taking the extracted yeast genome as a template, positive transformants were analyzed by PCR with P1 and P4 primers to verify the integration of the recombinant *L-Ag85B* fusion gene.

Expression of the L-Ag85B fusion protein in recombinant *P. pastoris* GS115

Expression experiments were conducted according to the procedure of Invitrogen's Pichia expression manual. Positive transformants were inoculated into 10 ml BMGY medium (Buffered Methanol-complex Medium, 100 mM potassium phosphate, pH 6.0, 13.4g/L YNB without amino acids, 20 g/L peptone, 10 g/L yeast extract, 0.4 mg/l biotin, and 10 g/L glycerol). The cultures were incubated at 30℃ with constant shaking at 220 rpm until the OD600 reached 4-6. The cells were harvested by centrifugation (3000 rpm, 5 min) and were then resuspended in 100 mL BMMY medium (the Buffered Glycerol-complex Medium is same as BMGY, but with 10 g/L methanol instead of glycerol). The cultures were then incubated at 30℃ with constant shaking at 220 rpm for 4 days. However, 1% methanol was added every 24 h. The cultures were harvested by centrifugation (7000 g, 5 min, 4℃) to analyze the expression of the fusion protein. Nonetheless, *P. pastoris* transformed with pPICZαC was used as a negative control and was treated similarly.

Analysis of protein production by SDS-PAGE and Western blot

The secretory proteins in the culture supernatants were subjected to SDS-PAGE. Proteins were stained with 0.2% Coomassie brilliant blue R-250 or were transferred electrophoretically onto poly-vinylidene fluoride transfer membranes for western blot analysis. L-Ag85B fusion proteins were detected using monoclonal anti-HbsAg or anti-Ag85B antibodies. Peroxidase-conjugated goat anti-mouse or anti-rabbit IgG were respectively used as the secondary antibodies, and in developing the Western Blots.

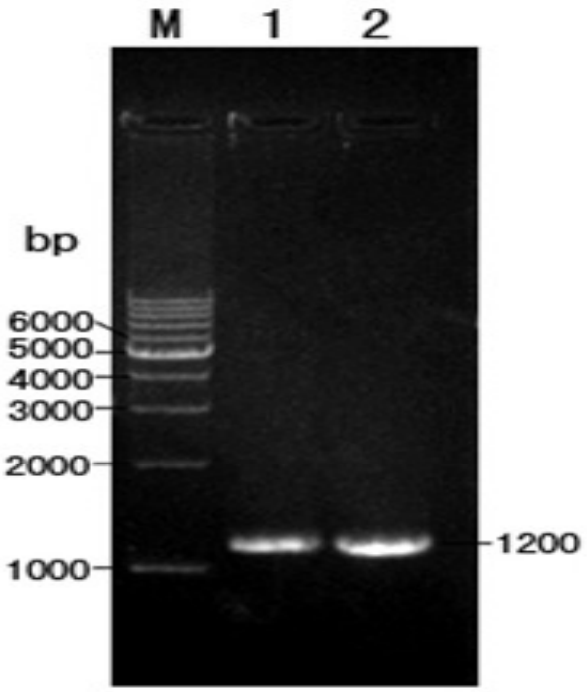

Figure 1. Agarose gel electrophoresis of the HBV *L* PCR product using primers P1 and P2 (lanes 1 and 2). M: 1 Kb DNA marker.

Detection of L-Ag85B fusion protein with serum samples by ELISA

After 4 days induction of the medium containing 1% methanol, the supernatant was collected, and after 56.8 g/100 mL (about 85% saturation at 4℃) ammonium sulfate precipitation, Y-PER* 6xHis Fusion Protein Purification Kit was then used to purify the protein. The purified protein L-Ag85B was used as coating antigen. In the serum samples, anti-HBsAg positive serum (convalescent hepatitis B patients) and anti-Ag85B serum (suspected TB patients) were used as the antibody sources. At the same time, the blank (saline) and healthy human serum was set as a negative control, while the mouse anti-HbsAg monoclonal antibody and rabbit anti-Ag85B monoclonal antibody was set as a positive control. Serum samples were diluted at 1:50, 1:100 and 1:200. When the samples were assayed at 450 nm with a microplate reader, the test OD value of every well was used to determine the level of serum antibody. Consequently, all experiments were performed in triplicate. When the OD values of the samples were more than 2.1 times as a negative control, the results were defined as positive.

RESULTS

Amplification of HBV L and MTB Ag85B gene by PCR

We used the HBV genome extracted from HBsAg positive serum and the genome of MTB H37Rv as templates to amplify the HBV *L* gene and the MTB *Ag85B* gene, respectively. Agarose gel electrophoresis showed that the size of the PCR products was about 1200 and 860 bp, in anticipation (Figures 1 and 2).

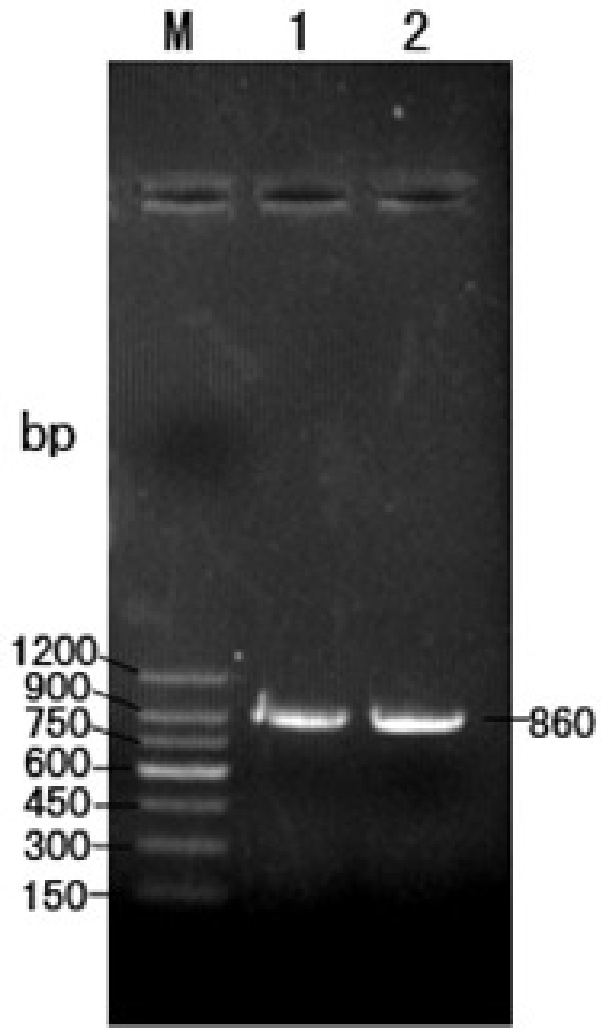

Figure 2. Agarose gel electrophoresis of the PCR product of *Ag85B* gene, using primers P3 and P4 (lanes 1 and 2). *Ag85B* gene PCR products are same with those anticipated. M: 150 bp DNA marker.

Colony PCR of pPICZαC-L-Ag85B plasmids

We used pPICZαC-L-Ag85B as a template, and P1 and P4 as primers to amplify *L-Ag85B* gene by PCR. As such, the agarose gel electrophoresis showed that the size of the PCR product was about 2060 bp in specific fragment, and the results were in conformity with the anticipated fragment (Figure 3).

pPICZαC-L-Ag85B plasmids detection by double restriction enzyme digestion

pPICZαC-L-Ag85B Plasmids were double digested by EcoRI and KpnI, and the agarose gel electrophoresis showed 2060 (*L-Ag85B* fusion gene) and 3600 bp (pPICZαC) in two specific fragments (Figure 4).

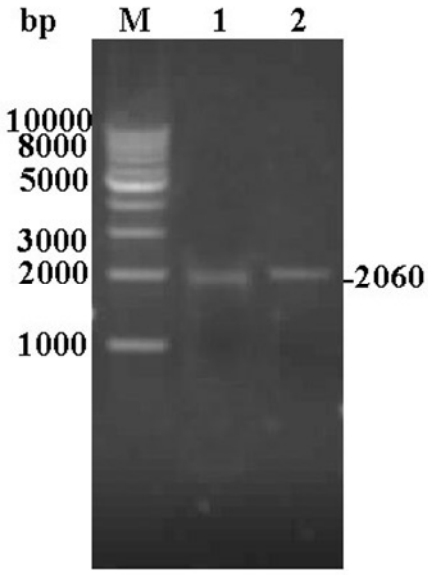

Figure 3. Agarose gel electrophoresis of the PCR product of *L-Ag85B* gene, using primers P1 and P4 at annealing temperature of 56℃. Lanes 1 and 2: *L-Ag85B* gene PCR products are same with those anticipated. M: 1 Kb DNA marker.

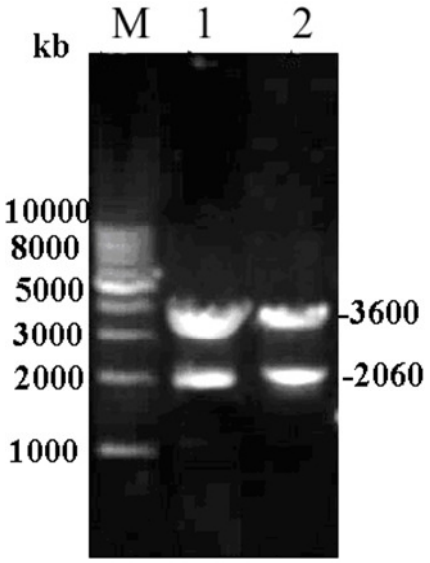

Figure 4. Agarose gel electrophoresis of the double digestion of pPICZαC-L-Ag85B with EcoRI and KpnI. Lanes 1 and 2: Two specific fragments, 2060 bp of *L-Ag85B* fusion gene and 3600 bp of pPICZαC vector. M: 1 Kb DNA Ladder Maker.

Sequencing identification of pPICZαC-L-Ag85B plasmids

The insertion of the recombinant plasmid pPICZαC-L-Ag85B was checked by sequencing, and the results showed that it was the same as reported by HBV *L* gene (GI: 157091234) and MTB *Ag85B* gene (GI: 6469794) sequences. It showed that the recombinant plasmid pPICZαC-L-Ag85B was successfully constructed.

Identification of recombinant yeast by PCR

We extracted yeast genomic DNA taken as a template for PCR with primers P1 and P4 to confirm the integration of *L-Ag85B*. A 2060 bp PCR product was detected in positive transformants. By agarose gel electrophoresis, the result was in agreement with *L-Ag85B* fragment. *L-Ag85B* was correctly integrated into the *P. pastoris* genome, which is desirable for the stable fusion protein production (Figure 5).

Assay of the protein recombinant yeast by SDS-PAGE and Western blot

SDS-PAGE analysis with the culture supernatants showed that the recombinant protein secreted from *P. pastoris* GS115 had a molecular weight of 78 KD (Figure 6). We detected the fusion protein by Western blot analysis, and it was proved that the protein can produce specific reaction with Mouse anti-human HbsAg\Ag85B monoclonal antibody (Figures 7 and 8).

Results of L-Ag85B fusion protein with serum samples by ELISA

The purified protein L-Ag85B was used as coating antigen (Figure 9). When serum samples were diluted at 1:50, the test results for 8 cases of anti-HBsAg positive serum of convalescent HB patients were all positive; at 1:100, 3 cases of serum were weakly positive, while others were negative; and at 1:200, all the cases were negative.

When the serum samples were diluted at 1:50, the results for 2 out of 8 cases of serum with suspected tuberculosis patients were weakly positive, while others were positive, but at 1:100 and 1:200 fold dilution, the results for all samples of serum were negative. The results show that the purified L-Ag85B fusion protein could maintain the specific immunoreactivity with anti-HBsAg positive serum and anti-Ag85B positive serum.

DISCUSSION

In recent years, series of problems have arisen in preventing various infectious diseases, such as higher frequency of children vaccination, more difficulties in vaccine management, greater cost of vaccination, etc

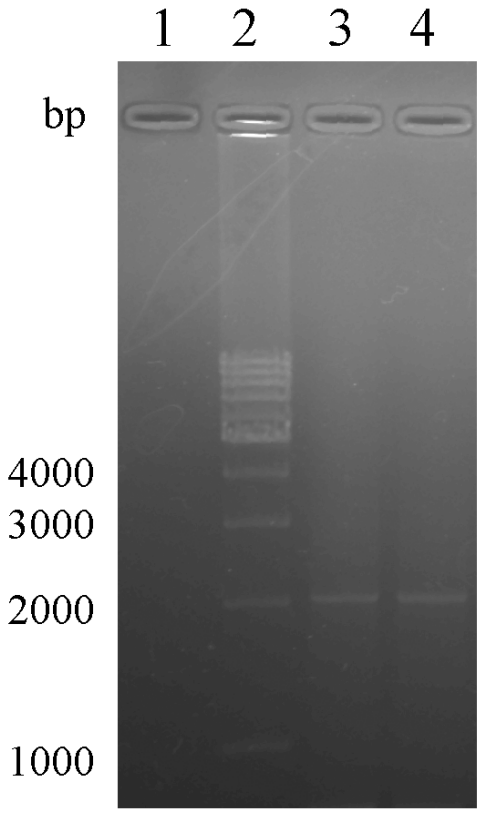

Figure 5. Agarose gel electrophoresis of the PCR product of *L-Ag85B* gene, using primers P1 and P4, at annealing temperature of 56°C for the identification of the recombinant yeast cells. Lane 1: PCR products from *P. pastoris* GS115 genomic DNA; lane 2: 1 Kb DNA marker; lanes 3 and 4: *L-Ag85B* gene PCR products from recombinant yeast genomic DNA.

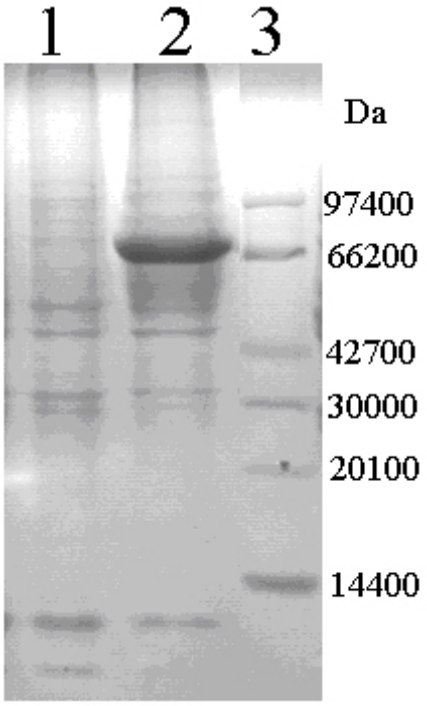

Figure 6. SDS-PAGE of the L-Ag85B fusion protein. A specific fragment of 78 KD was produced only in the recombinant yeast strain. Lane 1: The sample of the supernatant of *P. pastoris* GS115; lane 2: The sample of the supernatant of recombinant *P. pastoris* GS115; lane 3: protein marker.

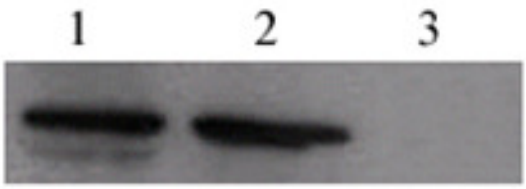

Figure 7. Western-blot of the L-Ag85B fusion protein probed with an HbsAg monoclonal antibody. Lanes 1 and 2: Lysate supernatant from the recombinant *P. pastoris* GS115; lane 3: Lysate supernatant of *P. pastoris* GS115.

(Wang and Hu, 2007; Yang et al., 2006). Therefore, it is imperative to develop and promote the application of a combined vaccine which can prevent various diseases (Einer-Jensen et al., 2009; Jing et al., 2002; Jin et al., 2006). Currently, the main component of the combined vaccine of HBV and TB are HBsAg and BCG. The combined vaccine is a hybrid solution, containing the engineering recombinant HBV vaccine and the attenuated BCG vaccine (Jin et al., 2006). The combined vaccine of HBV and TB is safe, but there are also defects of immunogenicity affecting its preventive effect. In addition, the hybrid solution of the two vaccines needs improvement, in its stability, dosage, prescription design and production costs, which has not been widely used in China. Scientists all over the world are still committed to developing new types of HBV-TB combined vaccine.

The HBV genome contains four ORFs, called S, C, P and X region. The S gene region includes three ATG initiation codons: PreS1, PreS2 and S segments, encoding three kinds of surface antigen protein. S protein is translated by the S region, M protein by the PreS2 and S region and the L protein by the PreS1, PreS2 and S regions (Young et al., 2001). Thus, when the L and S protein were compared, the L protein has PreS1 and PreS2. The 21-47aa of PreS1 act as the liver cell receptor binding site and the PreS1 peptide has many T cells and B cell epitopes, the immunogenicity of which is stronger than the S protein (Le Seyec et al., 1999; Cruciani et al., 2007; Rizza et al., 2002). The PreS2 (120-145aa) sequence also contains multiple epitopes and neutralizing sites of viruses. The vaccine preparation, when joined by PreS1 and PreS2 epitopes, can induce more efficiency in immune response, produce antibodies

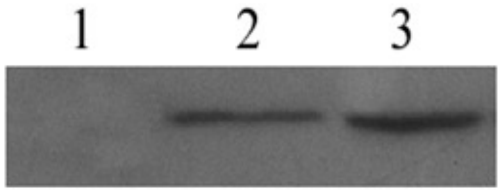

Figure 8. Western-blot of the L-Ag85B fusion protein probed with Ag85B monoclonal antibody. Lane 1: Lysate supernatant of *P. pastoris* GS115; lanes 2 and 3: Lysate supernatant from recombinant *P. pastoris* GS115.

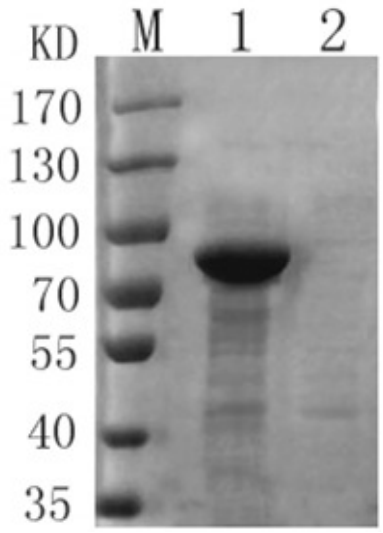

Figure 9. SDS-PAGE of the purified protein L-Ag85B. A specific fragment of 78 KD was produced only in the recombinant yeast strain. Lane 1: the purified protein L-Ag85B of the recombinant *P. pastoris* GS115; lane 2: the purified supernatant of *P. pastoris* GS115; lane 3: protein marker.

against HBV, protect organs against viruses, break immune tolerance and clear HBV. Moreover, the frequency of non-response or low-response to traditional vaccine could be greatly reduced (Sasaki et al., 2003; Pol et al., 2001).

Ag85B is a major early secreted protein of *M. tuberculosis* and BCG is a major protective antigen of *M. tuberculosis*. With nine immunodominant, Ag85B can induce a high level of specificity of the antibody response and the Th1-type cellular immune response (Skinner et al., 2003; Kariyone et al., 2003; Dietrich et al., 2006). It also has specific T cell recognition sites, which stimulate the production of memory T cells, promote cytokine secretion and enhance the activity of CTL (Elvang et al., 2009; Triccas et al., 2002). In addition, Ag85B protein easily binds human fibrin and promotes phagocyte *M. tuberculosis*, which thus plays a stronger role in the prevention of tuberculosis re-infection (Sinha et al., 1997). Studies show that the vaccines containing Ag85B gene enhance the immune effect of BCG (Skinner et al., 2003) and induce a stronger CTL activity (Zhang et al., 2006). More and more researches focus on the function of Ag58B, in that it is now used in human trials (Van Dissel et al., 2010). In this sense, Ag85B could be seen as an important antigen candidate for TB vaccine.

Pentapeptide as a linker, composed of glycine and serine (G-S-G-G-S), is added to the upstream primer of MTB Ag85B gene. The purpose of adding the linker to the fusion proteins is to make HBV L protein and Ag85B fold the best in their natural 3D structures and to prevent their intrinsic immunogenicity (Gustavsson et al., 2001). In studying the character of the fusion protein, the recombinant plasmid is transformed into *P. pastoris* GS115, and the expression of the L-Ag85B fusion protein is determined by SDS-PAGE and Western blot.

In recent years, *P. pastoris* has been developed quickly as one of the most important expression systems (Gellissen, 2000). This is due to the large scale of production of recombinant proteins with high expression levels and inexpensive growth media. Pichia is capable of performing posttranslational modifications such as glycosylation and proteolytic processing that aremore similar to human protein modifications. It is easier to purify heterologous proteins because *P. pastoris* releases few of its own proteins into the culture medium comparatively (Li et al., 2010). pPICZαC which is a yeast secretive expression vector, with a strong secretion signal peptide α-factor, can guide the protein secretion to extracellular, increase the production of foreign proteins and simplify its purification process.

In this study, hepatitis B *L* gene and tuberculosis *Ag85B* gene combined vaccines are successfully constructed and the fusion protein is expressed in *P. pastoris* GS115. Using the fusion gene in *P. pastoris* expression, a higher stability of the protein which can be modified for effective processing can be obtained. Detection of the purified L-Ag85B fusion protein with anti-HBsAg positive serum and anti-Ag85B positive serum by ELISA thereby provide a preliminary proof of the fusion protein that can maintain the specific immunoreactivity. In conclusion, this study successfully constructed the yeast expression system to the expression of L-Ag85B fusion protein, and provided a basis for the further study of hepatitis B and tuberculosis combined vaccine immunogenicity *in vivo* and immune protection.

ACKNOWLEDGEMENTS

This study was supported by the research fund from Science and Technology Plan of Guangdong Province No. [2006] A20101006, and the Major National Science

and Technology (S&T) Special Projects (2008ZX10002-009).

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