Iranian Journal of Veterinary Research, Shiraz University, 2013, Vol. 14, No. 3, Pages 211-219

Expression, purification and glycosylation analysis of chicken infectious bursal disease virus VP_2 in yeast

Cai, M.[1, 2]; Zhu, F.[3]; Wu, H.[4] and Shen, P.[1*]

[1]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210093, PR, China; [2]Department of Biotechnology, School of Food and Biology Engineering, Jiangsu University, Zhenjiang, Jiangsu 212013, PR, China; [3]MSc Student in Biology, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210093, PR, China; [4]Ph.D. Student in Biology, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210093, PR, China

[*]**Correspondence:** P. Shen, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210093, PR, China. E-mail: ppshen@nju.edu.cn

(Received 17 Jan 2012; revised version 4 Mar 2013; accepted 13 Mar 2013)

Summary

Infectious bursal disease (IBD), a highly contagious and devastating disease in young chicken, is caused by infectious bursal disease virus (IBDV). To improve the immunogenicity of recombinant IBDV subunit vaccine, an attempt was made to find a new way to prepare IBD vaccine containing glycosylated mVP_2 antigen. Firstly, IBDV mVP_2 gene (with a nucleic acid sequence encoding B cell epitope of IBDV (KFDQML) in the 5′-end of the VP_2, with a nucleic acid sequence encoding B cell epitope of IBDV (LASP) and (His) 6-tag in the 3′-end of the VP_2) was cloned. Secondly, IBDV mVP_2 protein was expressed in the methylotrophic yeast *Pichia pastori*s which can secret glycosylated protein. The recombinant mVP_2 protein could be stained pink with periodic acid-schiff reagents (PAS), which showed that mVP_2 was glycosylated. Finally, IBDV mVP_2 protein was purified with His-Trap (1 mL) affinity chromatography. These results indicate that glycosylated IBDV VP_2 protein modified with epitope peptides can be expressed in *Pichia pastoris*, which lay the groundwork for the development of a recombinant infectious bursal disease vaccine with high immunogenicity.

Key words: Infectious bursal disease, VP_2, Glycosylated, Epitope peptide

Introduction

Infectious bursal disease (IBD) is one of the serious diseases in chicken (Mahmooda *et al*., 2007), that has a worldwide distribution (Hsieh *et al*., 2007) and may cause a mortality rate of 30-60% in young chickens (Berg, 2000). The disease, caused by infectious bursal disease virus (IBDV), can induce a persistent immune-suppression. Infectious bursal disease virus can destroy B lymphocytes precursors in the bursal lymphoid follicles, which leads to poor immune response to other vaccination against pathogens. The IBDV capsid is formed by two major structural proteins, VP_2 *and* VP_3 (Castón *et al*., 2001). Serotype-specific antigenic epitopes that are responsible for eliciting neutralizing anti-bodies are located on VP_2 (Snyder *et al*., 1988; Mundt *et al*., 1995).

Vaccination against IBD with in-activated or live viruses plays an important role in preventing the disease (Mahmooda *et al*., 2007). However, these vaccines have some defects, such as repetitive injections, the probable transmission of vaccine strain of IBDV in the vaccine production and immunization process. Recent studies showed that different IBDV VP_2 antigen proteins of recombinant subunit vaccine against IBD were expressed in various hosts, such as *Escherichia coli* (Azad *et al*., 1991), baculovirus, plant (Wu *et al*., 2004) and *Pichia pastoris* (GS115) (Wang *et al*., 2003). Although recombinant vaccine with VP_2 antigen had been expressed in these hosts and had immunogenicity in chicken, it was not efficacious to protect chickens against the lesions of bursa of Fabricius

(Wang *et al*., 2003) or attack from IBDV antigenic variant strains. Although it is not exactly clear what causes these problems, low immunogenicity of VP_2 protein may be one main cause. Therefore, the veterinary medical world has been eagerly awaiting the development of high immunogenicity of IBD vaccines against vvIBDV.

Many experimental evidences indicated that epitope-vaccine could be a new strategy to develop effective vaccines for prevention and immunotherapy against viral infection (Xiao *et al*., 2001). Epitope-vaccines afford powerful approaches for eliciting potent and complete immune protection against not only primary homologous viral isolates but also heterologous viral mutants. They also can trigger T-cell-dependent Ig G antibody responses (Liu *et al*., 2003). Protein glycosylation plays an important role in the biological activity involved in antigen recognition in the innate and adaptive immune response (Rudd *et al*., 2001).

To increase the immunogenicity of recombinant IBDV subunit vaccine, glycosylated IBDV mVP_2 protein (with an epitope of IBDV (KFDQML) at the N terminal of VP_2, with an epitope of IBDV (LASP) and (His) 6-tag at the C terminal of VP_2), was expressed in the methylotrophic *Pichia pastoris* X-33. All these two epitopes are neutralizing B-cell epitopes of IBDV, which are recognized as key parts of the induction of humoral immune responses against target antigens.

Results in this study showed that IBDV mVP_2 protein could be expressed and modified by glycosylation in *Pichia pastoris* X-33. Recombinant mVP_2 protein secreted in the culture supernatant was purified by His-Trap (1 mL) affinity chromatography with purity greater than 95%. These results suggested that this research provided a new way for preparing IBD vaccine containing glycosylated VP_2 antigen.

Materials and Methods

Strains, vector, antibodies and chemicals reagents

Escherichia coli DH5a was used for the cloning of recombinant pPICZαA/IBDV-mVP_2 plasmid. The pPICZαA vector purchased from Invitrogen was used for secreted expression of IBDV mVP_2. The methylotrophic yeast *Pichas pastoris* strain X-33 (wild type Mut^+) was used for the integration of the pPICZαA/IBDV mVP_2 plasmid. The restriction enzymes and T4 DNA ligase were purchased from Takara and New England Biolabs, respectively. Rabbit anti-$(His)_6$-tag-polyclonal primary antibodies were purchased from Genesite. Goat anti-rabbit-IgG peroxidase-conjugated secondary antibodies were purchased from Santa Cruz and molecular weight standards for SDS-PAGE were obtained from Cell Signaling Technology. Yeast extract peptone dextrose (YPD) medium, buffered complex glycerol medium (BMGY) and buffered complex methanol medium (BMMY) were prepared according to the manufacturer's instructions. His Trap ^{TM} (1 mL) affinity column was purchased from GE-HEALTH. The pMD_{19}-T-VP_2 plasmid was a gift from Prof. Chen Puyan (Nanjing Agricultural University). The mVP_2 DNA was generated from the DNA of the pMD_{19}-T-VP_2, which includes the DNA sequence of infectious bursal disease virus VP_5 protein and $VP_{2\text{-}4\text{-}3}$ polyprotein genome (GI: 1296812).

Construction of recombinant expression plasmid

The full-length IBDV mVP_2-$(His)_6$ gene including about 1500 nucleotides was obtained by PCR amplification using one pair of primers: The forward primer was 5'-cgcgaattcatgaagtttgatcaaatgctgacgaacctgcaag atcaaacccaacagattgttccgttcatacgga-'3, which contains the nucleotide sequence (aagtttgatcaaatgctg) of epitope peptide (KFDQML) and an *Eco*R I site (underlined). The reverse primer was 5'-ccgtctagattaatgatgatgatgatgtggactagccaag gctcgagcagttcctga-'3, which contains the nucleotide sequence (tggactagccaa) of epitope peptide (LASP), an *Xba* I site (underlined) and 6 His tags at the 3'-end terminal of the amplified DNA. PCR reaction was performed using the following cycle conditions: 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min. The PCR amplified product was detected by electrophoresis on 1% agarose gel.

The purified IBDV mVP_2-$(His)_6$ DNA fragment was digested with *Eco*R I and *Xba* I and then cloned into the pPICZαA vector

cut with the same enzymes.

Transformation of X33 and PCR analysis of *Pichia* integrants

The expression plasmid pPICZαA-IBDV mVP_2-$(His)_6$ and pPICZα plasmid were linearized by Sac I. The latter was used as a negative control. Above two kinds of vectors were transferred into their respective yeast strain *Pichia pastoris* X33 by electroporation using a micropulser (Bio-Rad production, Gene Pulser X cell, USA) in a 0.2 cm cuvette. The electroporation procedure was carried out as follows: voltage, 1500 V; capacitance, 25 uF; resistance, 200 Ω; time, 5 ms and temperature, 0°C. The electroporated cells were grown on yeast extract peptone dextrose (YPD) agar medium containing 0.1, 0.2, 0.5 mg/mL Zeocin. To detect whether IBDV mVP_2-$(His)_6$ gene had been integrated to the yeast genome, 72 h later, three clones were picked and grown in YPD agar medium with different concentrations of antibiotics. The integrated transformants were confirmed by colony PCR using 5'AOX1 and 3'AOX1 PCR primers. The size of the PCR product is 588 bp using the genome isolated from the control. If the IBDV-mVP_2-$(His)_6$ gene was integrated into the *Pichia pastries* genome between the 5'AOX1 and 3'AOX1, the size of PCR product was approximately 2 kb.

Expression of IBDV-mVP_2 in *P. pastoris*

A single transformant integrated with pPICZaA-IBDV-mVP_2-$(His)_6$ was cultured in 25 mL BMGY (1% yeast extract, 2% tryptone, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol and 100 mM potassium phosphate pH = 6.0) in 250 mL capacity shake-flask in a shaking incubator at 28°C and 240 rpm. The culture media were harvested by centrifugation at 1500 g for 5 min at room temperature when the OD_{600} reached approximately 2. pPICZα A plasmid integrated transformant was also cultured as negative control. The sediment was then resuspended in 100 mL BMMY (1% yeast extract, 2% tryptone, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol and 100 mM potassium phosphate pH = 6.0). For sustained expression, methanol was added into BMMY media every 24 h to a final concentration of 0.5% (v/v). The 100 mL culture supernatant was collected by centrifugation (Eppendorf centrifuge 5810R, Germany) at 5000 rpm for 20 min in an F-34-6-38 rotor. After 96 h induction, all supernatants were collected for further study.

Western blot

The induced supernatants of the pPICZαA plasmid control and the transformants integrated with pPICZaA-IBDV-mVP_2-$(His)_6$ were analysed by 10% SDS-PAGE. Western blot was performed using polyclonal anti-His IgG (Genesite Biotech, China) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bioword Technology, USA). Then the blot was performed using the 3, 3, 5, 5-Tetramethyl Benzidine dihydrochloride (TMB) Chemiluminescence system (Cell Signalling, USA).

All the final induced supernatants of transformants were concentrated with Amicon Ultra (10 kDa) centrifugal filter unit to a final volume of 5 mL.

Purification of IBDV-VP_2 protein with His-Trap HP 1 mL column

The final enriched protein was filtrated through 0.22 μm filter to remove coarse particles and then purified with His-Trap HP 1 mL affinity column (GE Healthcare, England): His-Trap affinity column was washed with 5 column volumes of distilled water and equilibrated with 5 column volumes of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH = 7.4) to elute the IBDV-mVP_2 protein with $(His)_6$ tags. Then the eluted gradient was further subjected to dialysis with PBS in order to remove small molecule imidazole. In dialysis, PBS was exchanged every 8 h, two days later, the dialyzed IBDV-mVP_2 protein was collected and stored at -20°C.

Periodic acid-Schiff staining of recombinant IBDV-mVP_2 glyco-proteins (Trivedi *et al.*, 1983; Fang, 1998)

The proteins in the SDS-PAGE were

oxidized under periodically acidic condition (7 g/L, 5% (v/v) acetic acid, 1 h). After rinsing the gel three times with distilled water, it was put into the solution of 2 g/L sodium metabisulfite in 5% (v/v) acetic acid for 10 min. By removing the liquid and by adding 2 g/L sodium metabisulfite in 5% (v/v) acetic acid for 10 min again, the gel became achromatic. Subsequently the gel was maintained in 50 mL of the Schiff's reagent (basic fuchsin, sodium metabisulfite, and hydrochloric acid), and stored at 4°C for 1 h.

Results

Amplification of IBDV-m*VP*$_2$ gene

The PCR amplified IBDV-m*VP*$_2$-(His)$_6$ gene was about 1500 bp in size (Fig. 1a). To determine whether IBDV-m*VP*$_2$-(His)$_6$ gene had been linked to the yeast expression vector, pPICZαA, the constructed vector with two restriction endonucleases was digested, *Xba* I and *Eco*R I. Results in Fig. 1b show that the 1.5 kb DNA fragment appeared in the digested products, which confirmed that the expression plasmid pPICZαA-IBDV-m*VP*$_2$-(His)$_6$ had been successfully constructed.

IBDV-*VP*$_2$-(His)$_6$ protein contains 500 amino acids with 6 His tags at the C terminal (marked with a blue frame, Fig. 2) and three glycosylate sites (N-Ser/Thr) located dispersedly in the full-length *VP*$_2$ amino acid sequence (framed red, Fig. 2).

PCR analysis of *Pichia* integrants and western blot analysis

After pPICZαA-IBDV-m*VP*$_2$-(His)$_6$ has been obtained, the next thing should be whether the m*VP*$_2$ gene has been integrated to the yeast host cell genome and whether it could be expressed in this expression system. Then, the experiment would be continued as follows: PCR analysis of *Pichia* integrants could determine if the gene of interest has integrated into the *Pichia* genome. Results in Fig. 3a suggested that fragments, about 2 kb in size were obtained using the DNA of strain integrated IBDV-mVP2-(His)6 plasmid as the template. Moreover, approximately 580 bp nucleotides had been amplified using the strain genome integrated control plasmid as the PCR template (Fig. 3b). These figures show that the IBDV-m*VP*$_2$-(His)$_6$ and the control vector had been integrated to the genome of *Pichia pastoris*. To further examine the protein expression of inserted DNA sequence, the supernatants were harvested after induction of 72 h and then characterized by SDS-PAGE electrophoresis. Western blot analysis was done by using rabbit anti-His IgG antibody and mouse anti-rabbits IgG secondary antibody. As shown in Fig. 4, there are three additional protein bands on the western film instead of one, compared with the result of the control induced supernatant. These results suggest that the IBDV-m*VP*$_2$-(His)$_6$ gene can be expressed in *Pichia pastoris*, and the expressed m*VP*$_2$ proteins were of

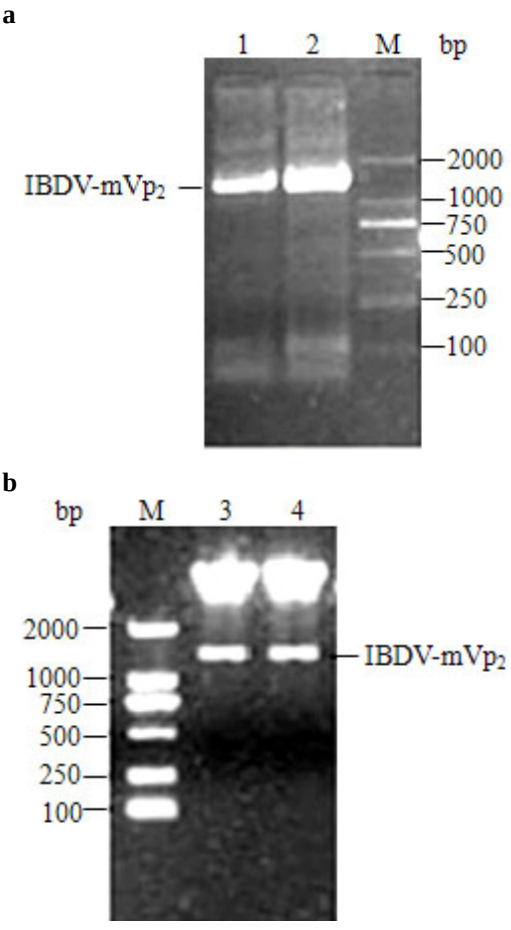

Fig. 1: Agarose gel electrophoresis analysis of IBDV-m*VP*$_2$ products (Fig. 1a) and detection of recombinant pICZa A-IBDV-m*VP*$_2$ (Fig. 1b). Lanes 1 and 2: Amplified m*VP*$_2$, Lane M: Standard DNA molecular weight marker, Lanes 3 and 4: pPICZaA-IBDV-m*VP*$_2$ digested by *Eco*R I and *Xba* I

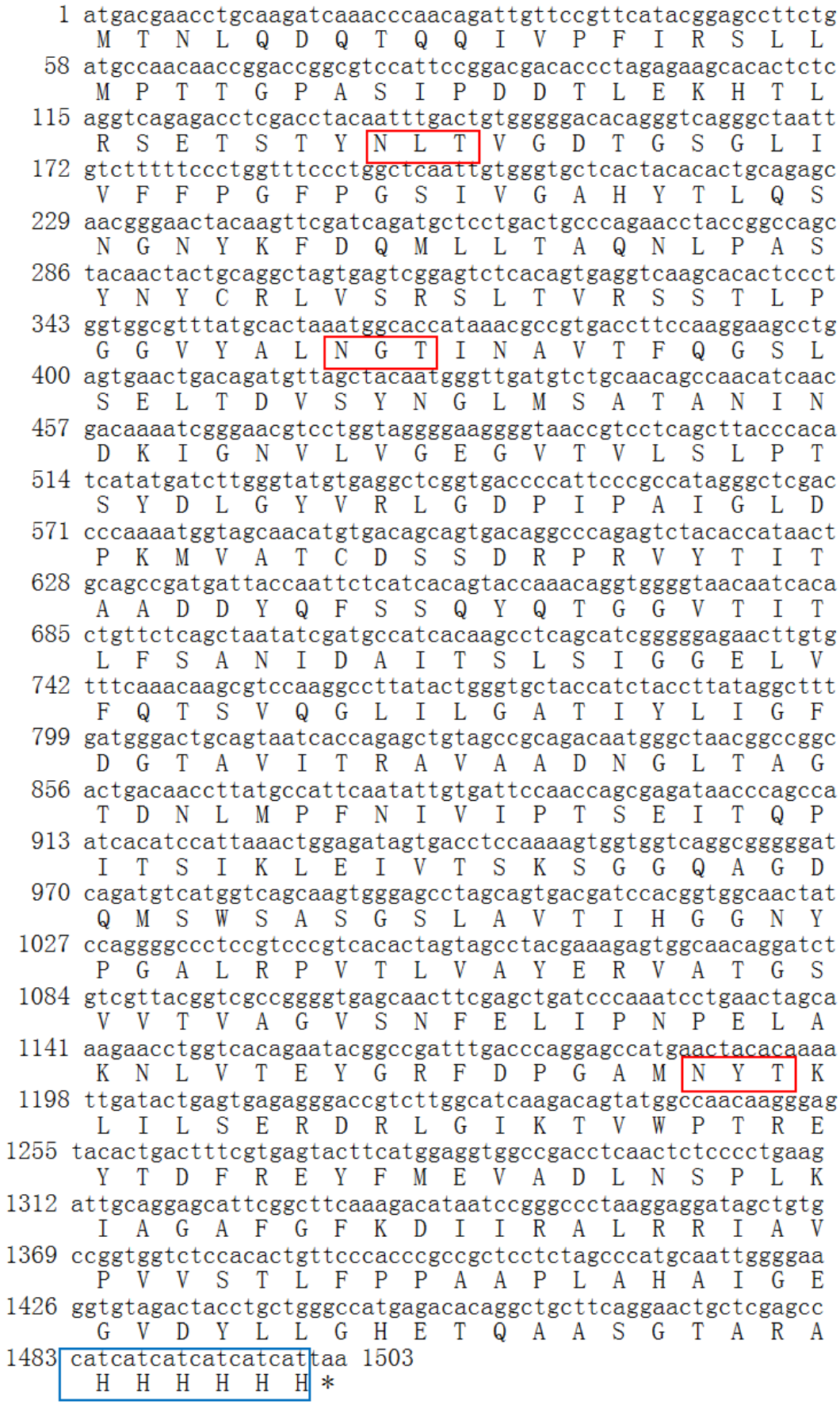

Fig. 2: The sequence of IBDV-*VP*$_2$ and its three glycosylate sites (red frame)

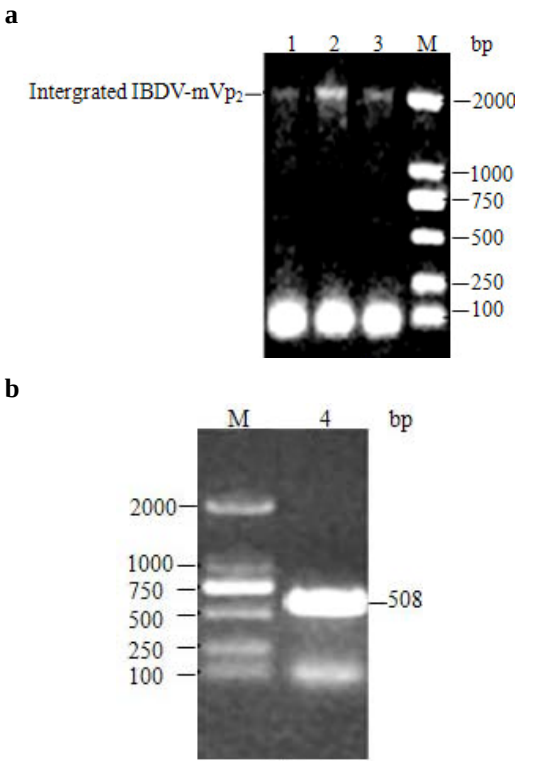

Fig. 3: Verification of IBDV-m*VP*$_2$ fragment integration to the host genome (Fig. 3a) and the control (Fig. 3b). Lanes 1, 2 and 3 show the PCR products of IBDV-m*VP*$_2$ fragment integrated to the host yeast genome. Lane 4: The PCR product of the control. Lane M: Molecular weight DNA ladder

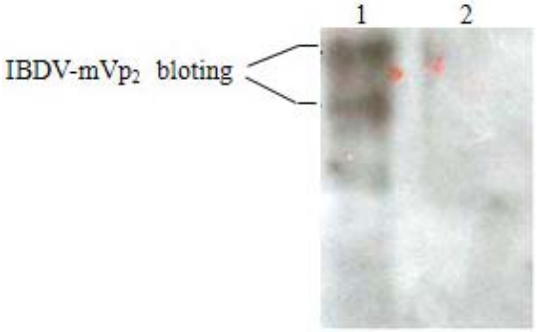

Fig. 4: Identification of m*VP*$_2$ by western blot using anti-his antiboby as primary antibody. Lane 1: The supernatants of IBDV-m*VP*$_2$ induced expressed at the point of 72 h, and Lane 2: The supernatants of the control

three kinds of molecular weight.

Purification and glycosylation of IBDV-m*VP*$_2$

The harvested supernatants containing the recombinant IBDV-m*VP*$_2$ were concentrated by Amicon Ultra 10 kDa to 5 mL, and then supernatants were detected by coomassie blue staining. As shown in Fig. 5a, there are seven protein bands in the SDS-PAGE. This result is different from western blot analysis due to the existence of other proteins secreted into the supernatants besides the IBDV-m*VP*$_2$ protein, which are unable to be detected by specific antibody in western blot analysis. Then the concentrated supernatants were purified by the His-Trap affinity chromatograph. The eluted fraction was analysed by 10% SDS-PAGE. Results in Fig. 5b show that the molecular weight of purified proteins are consistent with the aforementioned results (Fig. 4). These results confirm that those three proteins bands in the SDS-PAGE and the western blot film are chicken IBDV-m*VP*$_2$-(His)$_6$

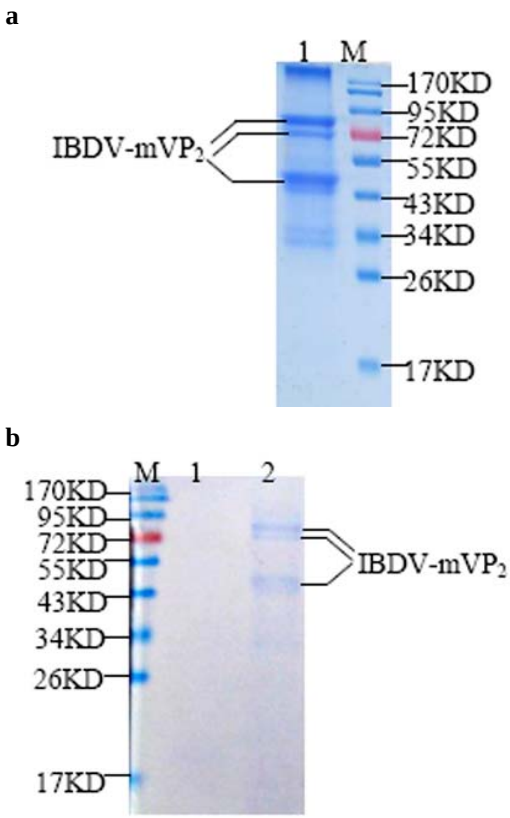

Fig. 5: Expression and purification of recombinant IBDV-m*VP*$_2$. a) Lane 1: Concentrated induced recombinant IBDV-m*VP*$_2$ supernatant. Lane M: Standard protein molecular weight marker. b) Lane 1: Concentrated induced control supernatant by the His-Trap affinity chromatograph. Lane 2: Concentrated induced recombinant IBDV-m*VP*$_2$ supernatant by the His-Trap affinity chromatograph. Lane M: Standard protein molecular weight markers

proteins expressed in *Pichia pastoris*. Now, a key question is why are there three different molecular weights of the same IBDV-mVP_2-$(His)_6$ protein? We considered that IBDV-mVP_2-6his protein expressed in the yeast might be glycosylated, which leads to the increase in the molecular weight.

Periodic acid-schiff staining of recombinant IBDV-mVP_2 glycoproteins

To confirm the above hypothesis, the purified recombinant IBDV-mVP_2 after His-Trap affinity chromatography was stained by periodic acid-schiff staining, by which glycosylated protein bands can be stained red. Results presented in Fig. 6a suggest that the upper two protein bands were stained as a whole distinct red area between 72-95 kDa, while the lower protein band showed light red between 43-55 kDa, consistent with the purified sample stained by coomassie brilliant blue in the SDS-PAGE (Fig. 6b).

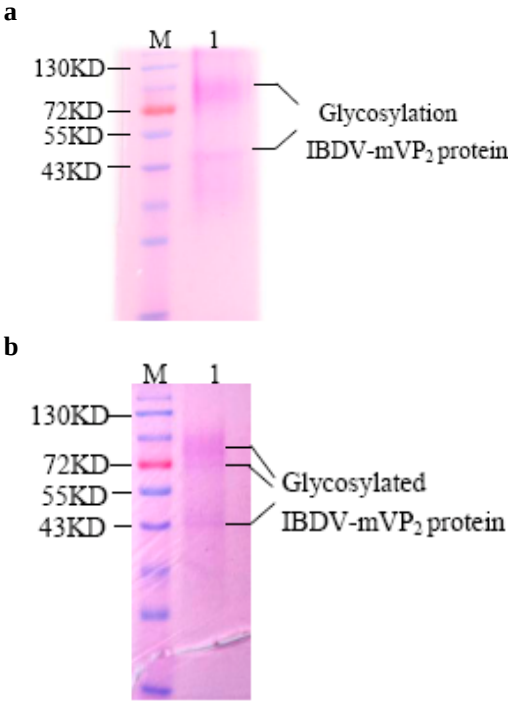

Fig. 6: Purified recombinant IBDV-mVP_2 by His-Trap affinity chromatograph was stained by periodic acid-schiff staining and coomassie blue. a) Lane M: Standard protein marker. Lane 1: The purified recombinant IBDV-mVP_2 that was stained by periodic acid-schiff staining. b) Lane M: Standard protein marker. Lane 1: The purified recombinant IBDV-mVP_2 that was stained by coomassie blue

Discussion

As no special drugs have been proved to be effective for treating chicken infectious bursal disease (IBD), vaccination of IBD is critical to chicken industries. Traditional attenuated or inactivated IBD vaccines play a critical role in the protection of the chicken against classical avian infectious bursal disease virus. However, they are not invasive enough to trigger a strong immune response to the variant strain of IBDV (vvIBDV) (Chettle *et al*., 1989; Chettle and Wyeth, 1989; Berg and Meulemans, 1991; Brown *et al*., 1994). Consequently, it is important to develop a new effective vaccine which can stimulate strong immune reaction to protect chicken against IBD infection.

There are many ways to enhance vaccines immunogenicity. For instance, we could use cytokines as adjuvant or increase the expression level of antigens. A recent report looks into improving the intrinsic immunogenicity of the antigen gene (Frelin *et al*., 2004), based on the rapid development of multi-epitope vaccines which showed immunological activity against virus, bacteria and cancers. In this study, antigenic epitope peptide codes were inserted into IBDV VP_2 gene at the two terminals of the gene nucleic acid sequence, respectively, and we hope to increase the immunogenicity, thus to trigger a stronger immune response.

Findings from our study confirm that chicken infectious bursal disease virus VP_2 antigen gene modified with epitope peptides codes (mVP_2) can be expressed in *Pichia pastoris* X-33. In addition, there are three glycosylated sites (Asn-X-Ser/Thr) (Fig. 2) in the mVP_2 gene sequence. Taking into consideration that it is a universal phenomenon that heterogeneous protein expressed in the methylotrophic yeast *Pichia pastoris* can be modified in glycosylation pathway into consideration, it is probable that the recombinant mVP_2 protein expressed in *Pichia pastoris* could be glycosylated. Results of periodic acid-schiff staining of recombinant IBDV-mVP_2 in this study also verified this glycosylation. As protein glycosylation plays a key role in the biological activity of the glycoproteins

involved in antigen recognition (Rudd *et al*., 2001), this work may offer a new way to produce recombinant IBD vaccine antigen, which may have some value in the prevention of IBD.

However, there are potential limitations in this study. During the experiment, we found that there is another pallid protein band in the SDS-PAGE, whose molecular weight is smaller than 50 kDa. But it can also be detected by anti-His antibody and His-Trap™ affinity column. The results imply that there might be degraded forms of m*VP*$_2$ in *Pichia pastoris* due to long induction time, as some proteins can be easily degraded by proteinase (Li and Zhang, 2006). Addition of some acid hydrolyzed casein to the yeast culture medium may be a good way to decrease the degradation of the recombinant m*VP*$_2$.

In brief, in this trial the m*VP*$_2$ gene was cloned, expressed, purified and also, as has been proved, glycosylated in yeast. Further research should be on animal testing of a new recombinant infectious bursal disease vaccine with the glycosylated recombinant m*VP*$_2$ protein for the prupose of providing solid evidence for developing a recombinant infectious bursal disease vaccine with high immunogenicity.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Project No. 31070764), the project for National Basic Science Personnel Training Fund (Project No. J1103512) and Jiangsu University Funds (Project No. 12JDG099). We would like to express our gratitude to Prof. Wang Yong (Jiangsu University) for polishing the language.

References

Azad, AA; McKern, NM; MacReadie, IG; Failla, P; Heine, HG; Chapman, A; Ward, CW and Fahey, KJ (1991). Physicochemical and immunological characterization of re-combinant host-protective antigen (*VP*$_2$) of infectious bursal disease virus. Vaccine. 10: 15-22.

Berg, TPV (2000). Acute infectious bursal disease in poultry: a review. Avian Pathol., 29: 175-190.

Berg, TPV and Meulemans, G (1991). Acute infectious bursal disease in poultry: protection afforded by maternally derived antibodies and interference with live vaccination. Avian Pathol., 20: 409-421.

Brown, MD; Green, P and Skinner, MA (1994). *VP*$_2$ sequences of recent European "very virulent" isolates of infectious bursal disease virus are closely related to each other but are distinct from those of classical strains. J. Gen. Virol., 75: 675-680.

Castón, JR; Martínez-Torrecuadrada, JL; Maraver, A; Lombardo, E; Rodríguez, JF; Casal, JI and Carrascosa, JL (2001). C terminus of infectious bursal disease virus major capsid protein *VP*$_2$ is involved in definition of the T number for capsid assembly. J. Virol., 75: 10815-10828.

Chettle, NJ; Stuart, JC and Wyeth, PJ (1989). Outbreak of virulent infectious bursal disease in East Anglia. Vet. Rec., 125: 271-272.

Chettle, NJ and Wyeth, PJ (1989). Failure of maternally derived infectious bursal disease antibodies to serotypes 1 and 2 to protect against heterologous virus. Br. Vet. J., 145: 165-169.

Fang, DF (1998). *The advancing technology of molecular biology*. 1st Edn., Beijing, Beijing Medical University and Peking Union Medical College Association Press. P: 26.

Frelin, L; Ahlén, G; Alheim, M; Weiland, O; Barnfield, C; Liljeström, P and Sällberg, M (2004). Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus nonstructural 3/4 A gene. Gene Therapy. 11: 522-533.

Hsieh, MK; Wu, CC and Lin, TL (2007). Priming with DNA vaccine and boosting with killed vaccine conferring protection of chickens against infectious bursal disease. Vaccine. 25: 5417-5427.

Liu, ZQ; Xiao, Y and Chen, YH (2003). Epitope-vaccine strategy against HIV-1: today and tomorrow. Immunobiology. 208: 423-428.

Li, ZL and Zhang, FC (2006). Progress of methylotrophic yeast *Pichia pastoris* expression system. Biotechnol. Bull., 6: 9-13.

Mahmooda, MS; Hussaina, I; Siddiquea, M; Akhtar, M and Ali, S (2007). DNA vaccination with *VP*$_2$ gene of very virulent infectious bursal disease virus (vvIBDV) delivered by transgenic *E. coli* DH5α given orally confers protective immune responses in chickens. Vaccine. 25: 7629-7635.

Mundt, E; Beyer, J and Muller, H (1995). Identification of a novel viral protein in infectious bursal disease virusinfected cells.

J. Gen. Virol., 76: 437-443.

Rudd, PM; Elliott, T; Cresswell, P; Wilson, IA and Dwek, RA (2001). Glycosylation and the immune system. Science. 33: 2369-2376.

Snyder, DB; Lana, DP; Savage, PK; Yancey, FS; Mengel, SA and Marquardt, WW (1988). Differentiation of infectious bursal disease viruses directly from infected tissues with neutralizing monoclonal antibodies: evidence of a major antigenic shift in recent field isolates. Avian Dis., 32: 535-539.

Trivedi, SM; Frondoza, CG and Humphrey, RL (1983). Modified technique for periodic acid-schiff staining of glycoproteins on agarose-film electrophoretograms. Clin. Chem., 29: 836-839.

Wang, XM; Wang, MP; Gao, HL; Gao, ZY; Zeng, XW and Zhang, SF (2003). High efficiency expression of the major antigen gene VP_2 of infectious bursal disease virus in yeast and rudimentary immunization test. Sci. Agr. Sinica., 36: 443-447.

Wu, H; Singh, NK; Locy, RD; Scissum, GK and Giambrone, JJ (2004). Expression of immunogenic VP_2 protein of infectious bursal disease virus in Arabidopsis thaliana. Biotechnol. Lett., 26: 787-792.

Xiao, Y; Lu, Y and Chen, YH (2001). Epitope-vaccine as a new strategy against HIV-1 mutation. Immunol. Lett., 77: 3-6.