

Expression of the VP2 gene of classical D78 infectious bursal disease virus in the methylotrophic yeast *Pichia pastoris* as a secretory protein

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ABSTRACT

Infectious bursal disease virus (IBDV) is the causative agent of Gumboro disease, an infectious disease of global economic importance in poultry. The expression of heterologous proteins in *P.pastoris* is fast, simple and inexpensive. In this study, VP2 encoding gene of classical D78 IBDV was amplified using reverse transcription (RT) polymerase chain reaction (PCR) and cloned into pPICZaA vector. Recombinant plasmid DNA was integrated into the chromosome of the transformed *Pichia pastoris* by electroporation and expressed protein identified by SDS- PAGE and western blotting. A recombinant protein was secreted into the supernatant from the yeast when induced with methanol. The expressed target protein in supernatant was bound with chicken anti IBDV Polyclonal antibodies. Western blotting with antibodies against D78 IBDV indicated that the recombinant VP2 protein retained its antigenicity. The concentration of secreted VP2 protein was 0.67mg/l.The production of recombinant VP2 protein indicated that *P. pastoris* was an efficient secreted expression system for D78 IBDV.

Keywords: IBDV, Secreted expression, VP2, Pichia pastoris

INTRODUCTION

Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens (kibenge *et al* 1988). The etiological agent, IBD virus (IBDV), has a predilection for the cells of the bursa of Fabricius where the virus infects actively dividing and differentiating lymphocytes of the B-cell lineage

(Burkhardte *et al* 1987). The chickens are susceptible to clinical disease at 3-6 weeks of age. The virus belongs to the family *Birnaviridae* of the genus Avibirnavirus (Murphy *et al* 1995). Members of the family contain a double stranded RNA genome consisting of 2 segments, designated A and B, within a non enveloped single–shelled icosahedral capsid of 60nm diameter. The IBDV genome segment A (3254bp) contains 2 open reading frames (ORF); small ORF Preceding and Partially

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overlapping the larger ORF encodes VP5 (Kibenge et al 1991). The larger ORF encodes a 109 KDa precursor polyprotein (N-VPx-VP4-VP3-c) which is processed into 2 structural proteins VP2(40-45KDa) and VP3 (32-34KDa) and the putative viral protease VP4 (28-30.5KDa) (Kibenge et al 1991). VP2 contains the major antigenic site responsible for eliciting neutralizing antibodies and VP3, the group specific site responsible for eliciting neutralizing antibodies specific antigens (Becht et al 1988) and a minor neutralize site (Jagadish and Azad 1991). In the last two decades many studies have been conducted in order to develop vaccines based on genetic engineering methods (Dertzbaugh 1998). VP2 expressed in Escherichia coli failed to promote neutralizing antibodies (Azad et al 1991, Omar et al 2006). On the other hand, immunization with VP2 expressed in yeast led to the formation of a high level of neutralizing antibodies that, when injected into specific-pathogen free (SPF) birds, conferred protection against wild IBDV challenge (Azad et al 1991).

The first commercial subunit vaccine was developed against hepatitis B virus and was expressed in Saccharomyces cervisiae (Valenzuela et al 1982). Another yeast expression system is the facultative yeast Pichia pastoris which utilizes methanol. The methanol metabolic pathway of P. pastoris, as in other methylotrophic yeasts, involves a unique set of enzymes. In the first step of this pathway, methanol is oxidised to generate formaldehyde and hydrogen peroxide which is then decomposed to water and molecular oxygen by catalase. The oxidation is carried out by two alcohol oxidase genes Aox1 and Aox2 (Cregg et al 1985). Aox1 is more active alcohol oxidase and may reach as much as 30% of the total Protein in the cell when cultured under growth limiting rates of methanol. This gene's promotor is utilized for expression of heterologous genes. The heterologous proteins in P. pastoris is fast, simple and inexpensive (Pitcovski et al 2003). Strong aerobic growth allows culturing at high cell densities. High levels of foreign protein expression have been shown for this vector and eukaryotic protein processing, modifications and folding can be performed. The objective of this work was the VP2 gene of D_{78} strain cloned, analyzed and expressed in a secreted yeast expression system.

MATERIALS AND METHODS

Virus and viral RNA Purification. The classical IBDV D_{78} strain (sequence in Genbank as IBDV D_{78} , the accession number AF499929) was grown in primary chicken embryo fibroblast (CEF). The fibroblast cells IBDV strain were derived from 10-day–old embryonated eggs and purified using methods described by Tsukamoto (Tsukamoto *et al* 1990). Total RNA from purified virus was extracted by using kit reagents, (Roche, Germany) according to the manufacture protocol. RNA samples were dissolved in nuclease free water.

Reverse transcription Polymerase chain reaction amplification. The VP2 of D78 was amplified with the forward and reverse primers of VP2 (Table 1) by RT- PCR Kit (One step-Titan, Roche) using a mixture of enzymes (superscript RT-PCR and expand high fidelity) in the following conditions 45 °C for 45 min and 94 °C for 2 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 1 min and 68 °C for 2 min and finally 72 °C for 10 min.

Table 1 . List of PCR Primers

Primer	DNA sequence
VP ₂ F	5´-GCCGGAATTCATGACAAACCTGCAAGAT-3´
VP ₂ R	5´-GCCGTCTAGAAACCTTATGGCCCGGAT-3´
$5-Aox_1$	5´-GACTGGTTCCAATTGACAAGC-3´
$3-Aox_1$	5'-GCAAATGGCATTCTGACATCC-3'
SD_1F	5´-TCAGGATTTGGGATCGC-3´
SD_1R	5´-CTCACCCCAGCGACCGATAACGACG-3´

Cloning of VP2 gene into pPICZaA vector. VP2 PCR products were separated in a 1% low melting agarose gel and purified by high pure PCR product purification kit (Roche). The specificity of the PCR

fragment was verified by nested PCR that reactions were 50 µl and contained 2 mM Mgcl2, 10 mM dNTPs, 10 Pmol of each primer SDF & R (Table 1), 2 µl RT-PCR product, 4 µl of PCR buffer and 1 unit of Taq DNA polymerase. The PCR program was as follows: 3 min at 94 °C, (30 sec at 94 °C, 60 sec at 55 °C, 2 min at 72 °C)×30 cycles and 10 min at 72 °C. The pPICZaA Vector (Easy Select Pichia Expression Kit, Invitrogen) and the resulting PCR fragments of 1355bp were purified from agarose gel were subsequently digested with EcorI and XbaI. The 1355bp insert (VP2) was ligated to digested pPICZaA. E. coli TOPIOF' cells were transformed with the resulting ligation products and plated on low salt LB/Zeocin medium containing 1% trypton, 0.5 % yeast extract, 0.5 % Nacl, pH 7.5 and 25 mg/ml zeocin (Leber et al 1999). The resulting transformants were tested by restriction analysis, and the positive clones were amplified to make larger amounts of DNA. The final DNA construct was linearized with MSSI restriction enzyme, the construct was transformed into the yeast P. pastoris electroporation according KM71H bv to manufactures protocol (Easy Select Kit, Invitrogen). The resulting electroporated cells were Plated on YPDS/ zeocin medium containing 1% yeast extract, 2% glucose, 1M sorbitol, and 100 Mg/ml zeocin. following the manufacture's instructions. Transformants bearing the chromosomally integrated copies of VP2 gene in cassettes were then detected by genomic PCR using the primers VP2 or 3' and 5' Aox1 primers (Table 1). Four positive clones of each transformants were used to inoculate 100 ml of buffered minimal glycerol medium (containing 100 mM potassium phosphate, pH 6, 13.4 g/liter of yeast nitrogen base without amino acids, 400 ng/lit biotin, 40 mg/liter Lhistidine, and 1% glycerol) overnight at 30 °C. The cells were then harvested and resuspended in 20 ml of buffered minimal methanol medium and incubated for 6 days at 30 °C. To induce expression, the culture were supplemented every 24 hrs with

methanol (100%) to a final concentration of 1% (v/v). 1 ml aliquots were withdrawn for cell viability determination and expression analysis.

SDS-PAGE and immunoblot analysis. Electrophoresis of the protein was performed as described by Laemmli (Laemmli, 1970), using 12% acryl amide gels followed by staining with coomassie blue or immunoblotting. For western blot analysis pellet and supernatant of samples were subjected to 7% sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) and transferred from the gels on to nitrocellulose membranes using buffer consisting of 25m MTris, 192 mM glycine, and 20% methanol. The membranes were blocked for one hour at room temperature in MPBST buffer (0.137 NaCl, 2.7 mM KCl, 8mM Na₂PO₄, 8 mM KH₂Po₄ 0.1%, tween20, and 5% Skimmed milk Powder). Subsequently, the membranes were incubated for one hour at room temperature with polyclonal chicken serum against IBDV (KPL, USA) were used at 1:200 dilution. Rabbit anti-chicken IgG labeled with HRP (Sigma, Germany) were used at 1:500 dilution was added to the membrane and incubated for 1h at room temperature. the membranes were washed 3x10 min with phosphate buffer saline (PBS) containing 0.01% tween20 and 2x10 min with PBS the immunoblot was visualized with DAB (3,3diaminobenzidine substrate) (Ausbel et al, 1999).

RESULTS

The VP2 was amplified by RT-PCR followed by PCR (Figure1A). The 1355bp fragment was obtained after amplification and by nested-PCR fragment in a 552bp, indicating the specificity of the PCR fragment (Figure 1B).

Cloning of VP2 in *E. Coli.* The 1355bp inserts were ligated into the multiple cloning site region downstream of the *P. pastoris* Aox1 promotor and the α -factor signal sequence of pPICZ α A vector using the *Ecorl / XbaI* restriction sites. The resulting

plasmids were transformed into the *E. coli* strain TOP10F'. The pPICZ α A plasmid contained the zeocin resistance gene for selection in *E. coli*. The construct digested with *EcorI* and *XbaI* (Figure 2).



Figure 1. A: PCR product of amplified VP2. *Lane 1*: amplified VP2 (1355bp); *Lane 2*: negative control, *Lane M*: molecular size markers (100bp). B: Nested PCR product of VP2. *Lane 1*: amplified SDI (552bp); *Lane M*: molecular size marker (100 bp).



Figure 2. Agarose gel electrophoresis of construct (VP2 + pPICZαA) digested by *EcorI* and *XbaI*. *Lane 1,3 and 5:* digested with *EcorI* and *XbaI* (construct); *Lane 2, 4 and 6*: not construct; *Lane M*: molecular size marker (1kb).

Expression of construct in yeast. The construct linearized with *MSSI* enzyme and following amplification of the plasmid transfected into yeast cells (KM71H strain).

Yeast transformants were detected by a genomic PCR assays using the Primers VP2 and the 3' and 5'Aox1 primers (Figure 3).



Figure 3. Typical genomic PCR assay of *P. pastoris* transformants on 1% agarose gel. A: genomic PCR analysis using 3'and 5'Aox1 primers. *Lanes 1 and 2*: positive clones. B: genomic PCR analysis using VP2 F and VP2 R primers. *Lanes 1-9*: positive clones (1355 bp); *Lane M:* molecular weight marker (1kb).

The VP2 protein was found in the supernatant and analyzed by SDS-PAGE (Figure 4A) and western blot. A band of the VP2 appeared at the same size as viral VP2 (Figure 4B). The concentration of secreted VP2 protein was 0.67 mg/l of the original fermentation volume.

DISCUSSION

As a major capsid protein of IBDV with multiple roles in IBDV evolution, VP2 has been the main target for many research groups around the world.VP2 is the major host- protective immunogen of IBDV.



Figure 4. A: Yeast transformation proteins in SDS-PAGE analysis shown in a representative coomasie blue-stained 12% polyacrylamide gel. *Lane1*: whole D78 IBDV; *Lane 2*: molecular mass standards; *Lane 3*: negative control; *Lane 4*: KM71H strain that transformed by pPICZ α A; *Lane 5*: KM71H strain that transformed by pPICZ α A; *Lane 5*: KM71H strain that transformed by pPICZ α A; *Lane 1*: negative control, yeast transformed with pPICZ α A. *Lane 2*: one clone of yeast VP₂. *Lane M*: molecular weight size markers.

At least three neutralizing epitopes have been identified on the VP2 protein. VP2 gene has been expressed in different expression systems. The P. pastoris expression system has gained acceptance as an important host organism for the production of foreign proteins. Generally, P. pastoris produces higher amount of heterologous proteins than other expression system and is particularly advantageous for smaller proteins (Cereghino & Cregg 2000). Protein expression in P. pastoris is based on the of the alcohol oxidase, gene Aox1.Transcription of the gene is regulated by the Aox1 coding sequence is replaced by a gene of interest. The P. pastoris expression system is a eukaryotic system. That is efficient and less expensive than expression in other eukaryotic system (e.g. mamalian, insect). This system has been used for the production of various recombinant proteins (Sreekrishna et al 1988). Furthermore it was shown that P. pastoris yeast is not pathogenic to chicken even administered live at a very high concentration (Pitcovski et al 2003). Recently, the use of yeast P. pastoris has resulted in

recombinant VP2 (rVP2) production level of up to 1 mg/l (Wu *et al* 2005).

In this study, we employed the *P. pastoris* expression secretional vector system to produce of rVP2 that concentration was 0.67mg/l. However, high levels of production necessitate some degree of optimization. As the first step to study the function of VP2 and develop a VP2 subunit vaccine for IBDV, we have expressed the VP2 gene of D78 strain in *P. pastoris*. Our yield definitively pointed out that the secreted yeast expression would be the choice for production of the VP2 protein. We are currently modifying cell growth conditions to optimize and obtain higher expression levels.

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References

- Ausbel, F.M., Brent, R., Kingston, R.E, Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1999). Short protocols in molecular Biology, 4th edn. Pp:..., Wiley, New York.
- Becht, H., Muller, H. and Muller, HK. (1998). Comparative studies on structural and antigenic properties of two serotypes of IBDV. *Journal Gene Virology* 69: 631-640.
- Burkhardt, E. and Muller, H. (1987). Susceptibility of chicken blood lymphoblasts and monocytes to IBDV. *Archives of Virology* 94: 297-303.
- Cereghino, J.L. and Cregg, J.M. (2000). Heterologus protein expression in the methylotrophic yeast *Pichia pastoris. FEMS Microbiology Review* 24: 45-66.
- Cregg, J.M., Barringer K.J., Hessler A.Y. and Madden, K.R. (1985). *Pichia pastoris* as a host system for transformations. *Molecular Cell Biology* 5: 3376-3385.
- Dertzbaugh, M.T. (1998). Genetically engineered vaccines: an overview. *Plasmid* 39: 100-113.
- Jagadish, M.N. and Azad, A.A. (1991). Localization of VP3 epitope of IBDV. *Virology* 184: 805-807.
- Kibenge, F.S.B., Dhillon, A.S. and Russel, R.G. (1988). Biochemistry and immunology of infectious bursal disease virus. *Journal Gene Virology* 69: 1757-1775.
- Kibenge, F.S.B., Mckenna, P.k. and Dybing J.K. (1991). Genomic cloning and analysis of the large RNA Segment (segment A) of a naturally a virulent serotype 2 IBDV. *Virology* 184: 437-440.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

- Leber, A., Hemmens, B. and Klosch, B. (1999). Characterization of Recombinant Human Endothelial Nitric – oxide synthase purified from the yeast *Pichia pastoris. Journal of Biological Chemistery* 274: 37655-64.
- Macreadic, I.G., Vaughan, P.R., Chapman A.J., et al. (1990). Passive protection against infectious bursal disease virus by viral VP2 expressed in yeast. *Vaccine* 8: 549-52.
- Murphy, F.A., Fauquet, C.M. and Bishop, D.H.L. (1995). Virus taxonomy. Classification and nomenclature of virus. Sixth Report of the International committee on Taxonomy of viruses. Pp:... (Archives of viral supplementary 10). Springer Verlag, New York.
- Nagarajan, M. and Kibenge, F. (1997). IBDV: A review of molecular Basis for variations in Antigenicity and virulence. *Canadian Journal of Veterinary Research* 61: 81-88.
- Pitcovski, J., Gutter, B. and Gallili, G. (2003). Development and large scale use of recombinant VP2 vaccine for the prevention of infectious bursal disease of chickens. *Vaccine* 21: 4736-4743.
- Sreekrishna, K., Patenz, R.H. and Cruze, J.A. (1988). High level expression of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Journal of Basic Microbiology* 28: 265-278.
- Tsukamoto, K., Obata, H. and Hihara, H. (1990). Improved preparation of ELISA antigen of IBDV. *Avian Diseases* 34: 209-213.
- Valenzuela, P., Medina, A., Rutter, W.J. and Ammerer, H. (1982). Synthesis and assembly of hepatitis B Virus surface antigen particles in yeast. *Nature* 298: 347-350.
- Wu P.C., Su, H.Y., Lee, L.H., et al. (2005). Secreted expression of the VP2 protein of very virulent infectious bursal disease virus in the methylotrophic yeast *pichia pastoris*. Journal of Virological Methods 123: 221-225.