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# Screening of Ginseng Proteins Interacted with BVDV E0 Protein

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**Abstract:** Bovine Viral Diarrhea Virus (BVDV), a single-stranded RNA virus can cause fatal diarrhoea syndrome, respiratory problems and reproductive disorder in herd. However, there still no effective vaccine or medicines for controlling the spreading of BVDV. In this study, researchers are interested in obtaining proteins with anti-BVDV activity in ginseng. The structure protein E0 of BVDV was used as bait protein to screen the ginseng cDNA library by the yeast two hybrid assay system. The result showed that eukaryotic translation initiation factor 5A (eIF5A) of ginseng has direct interaction with E0. The interaction was verified by GST-pull down analysis. This interaction provides a target for researching of antiviral drugs for controlling the spreading of BVDV.

**Key words:** Bovine viral diarrhea virus, ginseng, yeast two hybrid, cDNA library, reproductive disorder

## INTRODUCTION

Bovine Viral Diarrhea (BVD) is a widespread cattle disease. The causative agent is the Bovine Viral Diarrhea Virus (BVDV), a positive-stranded RNA virus of the family Flaviviridae (Gunn *et al*., 2005; Schweizer *et al*., 2006). BVDV infection presents a wide spectrum of disease, ranging from mild acute infection to fatal mucosal disease (Wenzhi *et al*., 2011). BVDV could transmit through the dam to the fetus before 125-182 days of gestation which leads to bovine fetus become immunotolerant to the infecting of BVDV resulting in a life long persistent infection (Ellsworth *et al*., 2006). Persistently infected animals serve as an effective source of viral transmission (Ames, 2005; Fulton *et al*., 2006). Also, BVDV could causes immunosuppression of the adaptive immune response which makes the animals more susceptible to other disease (Nettleton and Entrican, 1995). The two fundamentally different types of infection cause significant loss in the live-stock industry (Peterhans *et al*., 2006; Al-Haddawi *et al*., 2007; Berends *et al*., 2008). However, there still no effective vaccine or medicines for controlling the spreading of BVDV.

In recent years, BVDV has a rising trend in the transmission. Different strategies are available to control the spread of BVDV in a herd such as vaccination and test and cull schemes. Among these strategies, test and cull schemes have been very successful applied in areas with low cattle densities and low BVDV prevalence such as Scandinavia (Greiser-Wilke *et al*., 2003; Viet *et al*., 2006). But in the areas with high cattle densities and high BVDV prevalence, the program will bring huge economic loss.

As a promising method, vaccination can provide some protection from acute disease and the development of persistently infected foetuses (Moennig *et al*., 2005; Thomas *et al*., 2009). However, vaccination programmes alone cannot control or eliminate BVDV absolutely. The research of effective medicines to control the spreading of BVDV is of great value.

The genomes of BVDV is translated and processed into 11 mature protein, namely Capsid protein (C), N-terminalprotease (Npro), three glycoproteins (E1, E2, E0), protein of 7 kDa (p7) and five non-structural proteins ($NS_{2-3}$, NS4A, NS4B, NS5A, NS5B) (Thiel *et al*., 1996). E0 is an essential structural component of pestivirus particles but is also secreted from infected cells (Schneider *et al*., 1993). Glycoprotein E0 could inhibit IFN expression in infected cells (Matzener *et al*., 2009). The RNase activity of E0 is required for this inhibition and E0 could degrade ds and ssRNA at neutral pH. E0 also influence the activity of cell apoptosis induced by dsRNA (Magkouras *et al*., 2008; Schweizer and Peterhans, 2001). Those probably contribute to the survival of persistently BVDV-infected animals and maintains viral. Thus, E0 is a promising target for searching drugs to control the spreading of BVDV.

Traditional Chinese medicine has a long history in China. Many kinds of traditional Chinese medicine showed strong antiviral activity such as *Panax ginseng* C.A. Mey., Astragalus, Radix Glycyrrhizae and Herba Houttuyniae (Jiang *et al*., 2010; Zhang and Chen, 2008). Ginseng (*Panax ginseng* C.A. Meyer) is a medicinal plant used worldwide and has been reported to have various biological effects (Xie *et al*., 2005). It is reported that ginseng contains a lot of biologically active components

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like ginsenosides, polyacetylenes, acidic polysaccharides, ginseng proteins and phenolic compounds. Ginsenosides, polyacetylenes and ginseng proteins are the primary components of Asian ginseng for antiviral activity. Protein and polypeptide drugs with the advantages of high biological activity and target specificity have become research focus (Colao *et al*., 2002). As a kind of active components of ginseng, ginseng proteins show high antiviral activity (Ng and Wang, 2001; Chunzhi *et al*., 2001). Thus, searching for antiviral protein in ginseng has important value for inhibiting the spreading of virus infection such as BVDV.

In this study, researchers are interested in obtaining proteins with anti-BVDV activity in ginseng. The E0 of BVDV was used as bait protein to screen the ginseng cDNA library by the yeast two hybrid assay system. The protein to protein interaction was demonstrated by GST-pull down analysis.

## MATERIALS AND METHODS

Yeast strains AH109 and Y187 were purchased from clotech. The plasmid pMD 18T-E0 was got from the laboratory. The *E. coli* strains DH5α was purchased from Invitrogen (Novagen, China). Restriction enzymes, Taq DNA polymerase, TriPure kit and T4 ligase were purchase from TaKaRa Biotechnology Co. The 6 years old ginseng (*Panax ginseng* C.A. Meyer) was cultured in Jilin Agricultural University.

**Plasmid construct and auto-activation analysis of the pGBKT7-E0 plasmid:** The full length BVDV E0 cDNA was subcloned into pGBKT7 (Clontech) containing a GAL4 DNA-binding domain for the preparation of bait vector. Briefly, the BVDV E0 open reading frame was amplified from plasmid pMD18-T-E0 which contains the complete gene with the forward primer (5' CGGCCATG GAA AACATAACACAGT GGAACCT3', NcoI site underlined) and the reverse primer (5' CGGTCGACTTAAG CGTATGCTCCAAA CCACG3', Sal I site underlined). The PCR product was digested with Bam HI and Sac I and subcloned into pGBKT7 vector digested with the same enzymes to produce the bait plasmid pGBKT7-E0. The correct reading frame was confirmed by sequence analysis. Then, the plasmid was inserted into the yeast strain Y2H Gold using Lithium Acetate Method according to the Yeast Protocols Handbook (Clontech) and the transformants were selected on a synthetic medium lacking Trp. Auto-activation of E0 was analyzed by β-galactosidase assay using a filter-based assay.

**Library construction:** Total RNAs were extracted from ginseng roots using RNAiso reagents ((TaKaRa) and mRNAs were obtained by Ploy A Tract® mRNA Isolation system (Promega). The expression cDNA library was made by the SMART Method according to Make Your Own Mate and Plate™ Library System User Manual. To remove fragments smaller than 300 bp, the ds-cDNA was purified with CHROMA SPIN_TE-1000 Column (Clontech). The resulting cDNAs were cloned into pGADT7 vector and introduced into yeast strain Y187.

**Library screening:** Ginseng cDNA library was screened with bait plasmid pGBKT7-E0 according to the MATCHMAKER two-hybrid system (Clontech) user manual. Diploids were plated on quadruple dropout medium (SD/-Ade/-His/-Leu/-Trp) and incubated for 3-5 days at 30°C. The fresh growing clones were used for β-galactosidase assay as previous described (Pewe *et al*., 2005). Briefly, yeast was permeabilized by freezing yeast impregnated filters in liquid nitrogen and thawing at room temperature. This filter was placed over a second filter that was pre-soaked in Z buffer (pH 7.0). The liquid β-galactosidase activity was determined using the substrate O-Nitrophenyl β-D-Galacto-Pyranoside (OPNG). Positive and negative control group was set up. Three replicates were made in all determinations and Analysis of Variance (ANOVA) was performed in each test. The positive clones were characterized by sequencing and these sequences were analyzed by BLAST.

**Confirmation of interaction between full length eIF5A and E0 by yeast two-hybrid assay:** The full length cDNA of eIF5A was amplified by PCR from pGADT7-eIF5A and subcloned into pGADT7. The pGBKT7-E0 and pGADT7-eIF5A were transformed into Y2H Gold strain. The strain was plated on quadruple dropout medium (SD/-Ade/-His/-Leu/-Trp/X-α-gal) and incubated for 3-5 days at 30°C.

**Glutathione S-transferase pull-down assay:** The full length cDNA encoding E0 was amplified by PCR from pGBKT7-E0 and subcloned into pGEX-6T-1 (clontch). The ginseng *eIF5A* gene was amplified by RT-PCR and inserted into PET32α (clontch). The recombinant protein GST-E0 and protein eFI5A-His were individually expressed in *E. coli* BL21 cell. The GST pull down assay was carried out according to the MagneGST™ Pull-Down System protocol. Briefly, the purified GST-E0 protein was captured on the MagneGST™ Particles for 30 min and washed five times with MagneGST™ Binding/Wash Buffer. Then the pur purified His-eIF5A protein was added to MagneGST™ Particles and incubated at room temperature for 1 h. The particles were collected and washed five times. After boiling with SDS-PAGE sample

buffer, the bound proteins were analyzed by the bound proteins were analyzed by 15% SDS-PAGE followed by Western blotting using anti-His antibody. His-eIF5A protein was used as controls.

## RESULTS

**A novel binding partner of E0 is discovered by yeast two-hybrid screening assay:** To search for proteins that interact with the E0 protein in ginseng, the yeast two-hybrid system was used. The recombinant plasmid pGBKT7-E0 that had no auto-activation was used as bait vector to screen the ginseng cDNA library. All prey clones passed two rounds of screening in an attempt to minimize clonally specific false positives. After that, four positive clones were used for β-galactosidase assay (Fig. 1a). Here, a pGADT7-positive plasmid containing an 834 bp insert was chosen for further study. The cloned cDNA contained an open reading frame with 148 aa. Amino acid sequence analysis showed high homology with eukaryotic translation Initiation Factor 5A (eIF5A) of other species, especially at the region surrounding the hypusine modification site in the N-terminal part (Fig. 1b). The corresponding gene in ginseng was not reported.

**The interaction between full length eIF5A and E0 is confirmed by yeast two-hybrid assay:** Researchers used yeast two-hybrid assay to confirm the interaction between the full length eIF5A and E0. The full length ginseng eIF5A was subcloned into pGADT7. The two recombinant plasmids pGADT7-eIF5A and pGBKT7-E0 had no auto-activation. The coloration analysis showed only co-transforming pGADT7-eIF5A and pGBKT7-E0 could activate the reporter gene (Fig. 2). The results demonstrate that the full length eIF5A interacts with E0.

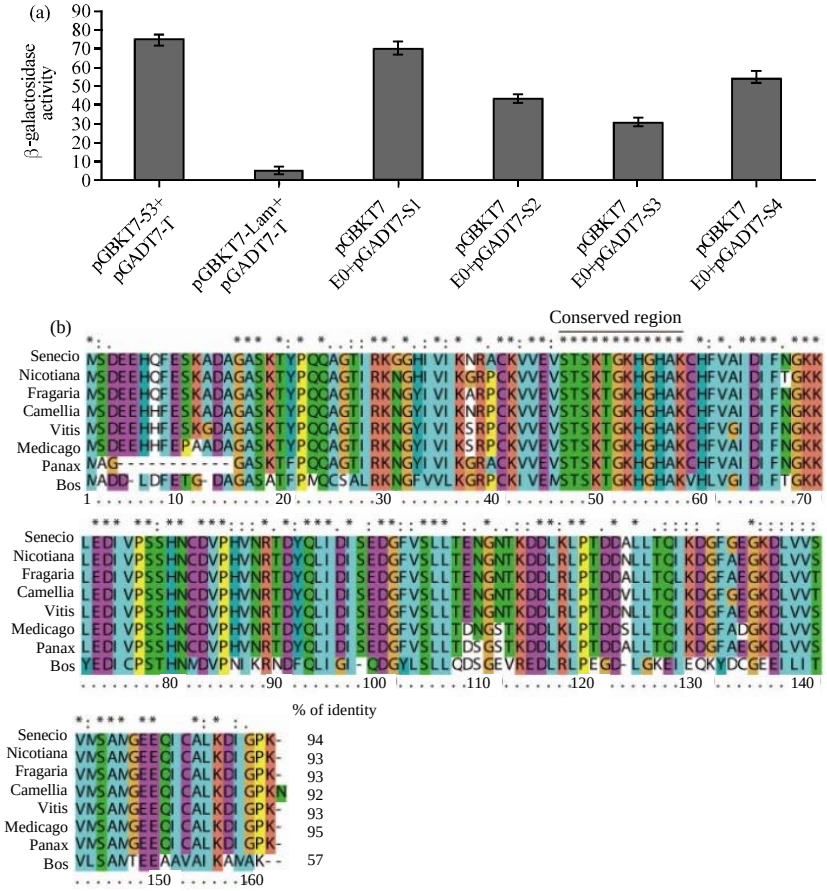


Fig. 1: β-galactosidase assay and sequencing of positive clones. a) Liquid β-galactosidase assay results. The β-galactosidase assay (intriplicate) were carried out by a liquid. The error bars represent standard error of the mean. b) Alignment of the amino acid sequences of eIF5A from *Medicago sativa*, *Fragaria vesca* subsp, *Senecio vernalis*, *Nicotiana plumbaginifolia*, *Camellia sinensis*, *Vitis vinifera* and *Bos taurus*. Gaps (-) were introduced to optimize alignment. Sequences identical to ginseng eIF5A are shaded

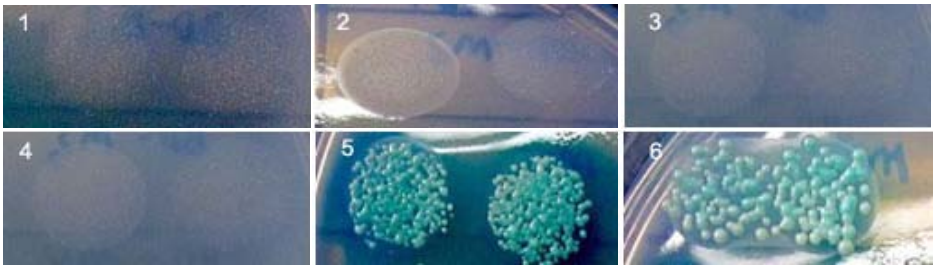


Fig. 2: Coloration analysis to verify the interaction between full length eIF5A and E0. 1: Co-transform pGBKT7-E0 and pGADT7-T; 2: Co-transform pGBKT7 and pGADT7-EIF5A; 3: Co-transform pGBKT7 and pGADT7-T; 4: negative control, pGBKT7-Lam + pGADT7-T; 5: Co-transform pGBKT7-E0 and pGADT7-EIF5A; 6: positive control, pGBKT7-53 + pGADT7-T

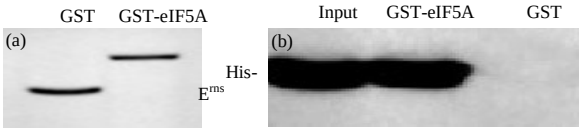


Fig. 3: GST pull-down assay demonstrating *in vitro* binding between E0 and eIF5A. a) Purification of GST-eIF5A protein. Purification of GST protein (Lane 1) and GST-eIF5A protein (Lane 2) by Western blot analysis using anti-GST monoclonal antibody. b) GST pull-down assay. Lane 1 demonstrated the signal from purified His-E0. GST protein and GST-eIF5A fusion protein were mixed with purified His-E0 (Lanes 2 and 3) and precipitated with glutathione agarose followed by Western blot analysis

**The direct interaction between E0 and eIF5A *in vitro* is verified by GST pull-down assay:** To verify whether eIF5A could bind E0 an *in vitro* GST-pull down approach was employed with the purified GST-eIF5A and GST protein. His-E0 protein was prepared from *in vitro* transcription/translation reaction and then individually mixed with glutathione agarose bound to either GST protein or the GST-eIF5A fusion proteins. As seen in Fig. 3, GST-eIF5A fusion protein interacts with His-E0 proteins. No such interaction was observed between GST protein and mutant His-E0 protein. Thus, the results demonstrate the direct interaction between eIF5A and E0 observed in the yeast two-hybrid screen.

## DISCUSSION

In this study, researchers successfully got the ginseng protein interacted with E0 of BVDV. The protein to protein interaction was demonstrated by GST-pull down analysis. The result showed that the eIF5A had directly interaction with the E0 of BVDV. The eIF5A is the only protein known to contain the unusual amino acid residue hypusine (Chen and Liu, 1997; Wolff *et al*., 2007). The translation factor eIF5A is essential for cell viability but the critical cellular role of eIF5A remains unclear. Some reports show that eIF5A was closely related with the virus infection. *eIF5A* gene expression is constitutively low but inducible with T-lymphocyte-specific stimuli in human peripheral blood mononuclear cells (Bevee *et al*., 1994). In contrast, eIF5A was constitutively expressed at high levels in virus-infected human cells. This show that eIF5A may interacted with T-lymphocyte to protect the cell from virus infection.

On the other hand, eIF5A is a conserve protein and deemed to be present in all eukaryotic cells (Schnier *et al*., 1991). The eIF5A of ginseng is showed 57% homology with that of bovine.

## CONCLUSION

Some reports show that eIF5A is the cofactor indispensable for Rex and Rev functions (Rex and Rev are necessary for the expression of structural proteins of human T-cell leukemia virus type I and human immunodeficiency virus type 1) (Bevec *et al*., 1996; Katahira *et al*., 1995). These indicate that eIF5A can participate in full playing of the retroviral protein function. BVDV is a positive-stranded RNA virus and the interaction between the eIF5A and E0 of BVDV further demonstrated the eIF5A may have close relationship with biosynthesis and transportation of BVDV structure proteins. This interaction provides a target for researching of antiviral drugs. Further biochemical studies should be down to elucidate function of eIF5A in cells infected by RNA virus.

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