การโคลนยีนและผลิตโปรตีนส่วน Antigenic domain ของไกลโคโปรตีนจีอี ของเชื้อไวรัสพิษสุนัขบ้าเทียมในเชื้ออีโคไลและการนำมาประยุกต์ ในการตรวจทางซีรั่มวิทยา

CLONING AND EXPRESSION OF AN ANTIGENIC DOMAIN OF GLYCOPROTEIN gE OF PSEUDORABIES VIRUS IN *Escherichia coli* AND ITS APPLICATION FOR SEROLOGICAL ASSAY

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บทคัดย่อ

ส่วนท้ายของยีนไกลโคโปรตีนจีอีของเชื้อไวรัสพิษสุนัขบ้าเทียมสายพันธุ์ 396 ถูกนำมาโคลนและผลิตโปรตีนในเชื้ออีโคไล ทำให้สามารถที่จะผลิตโปรตีนจีอีในปริมาณมากๆ และนำมาผลิตเป็นโพลีโคนอล แอนติบอดี โดยโปรตีนที่ผลิตได้ ประกอบด้วยกรดอะมิโนที่อยู่ตำแหน่ง 54-90 ของยีนและเชื่อมติดกับโปรตีน 6xHis-tagged proteins และ DHFR ใน pQE40 (QIAGEN) จากการเหนี่ยวนำการผลิตโปรตีนโดยใช้ IPTG พบว่าโปรตีนจีอีมีขนาดประมาณ 26.5 kDa ซึ่งปริมาณที่ผลิตได้ประมาณ 20% ของโปรตีนทั้งหมดที่ผลิตในอีโคไลและเมื่อผลิตเป็นโพลีโคนอล แอนติบอดีในหนู Balb/C และนำมาทดสอบทางซีรั่มวิทยาโดยวิธี IPMA และ IFA พบว่าสามารถแยกวินิจฉัยเซลเพาะเลี้ยงที่ติดเชื้อไวรัสพิษสุนัขบ้าเทียมสายพันธุ์ท้องถิ่น (396) ออกจากสายพันธุ์วัคซีน (gE$^-$) โดยไม่พบ cross reaction เชื้อไวรัสอหิวาต์สุกรและพีอาร์อาร์เอส

(คำสำคัญ: ไกลโคโปรตีนจีอี, เชื้อไวรัสพิษสุนัขบ้าเทียม, การโคลนยีน, การแสดงออกของยีน, วิธีทางซีรั่มวิทยา)

ABSTRACT

To produce a large quatity of functional gE protein for producing a polyclonal antibodies, an *E. coli* expression system containing the distal region of glycoprotein gE of PR virus (PR-gE) gene of the PR virus stain 396 was construct. The expressed protein contained 37 amino acids (54-90 amino acid fragment) fused with 19-amino acids tag containing 6 histidine residue and mouse DHFR. After induction, the rPR virus-gE polypeptide of 26.5 kDa was expressed to about 20% of the total *E .coli* proteins. Results of immunological assay (IPMA and IFA) indicated that the polyclonal antibodies specific for rPR virus-gE protein could differentiate wild type and vaccine strain PR virus infected cell line, without cross reaction with classical swine fever virus and porcine reproductive and respiratory syndrome virus.
(Keywords: Glycoprotein gE, Pseudorabies virus, Gene cloning, Gene expression, Serological assay)

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INTRODUCTION

Pseudorabies (PR) virus or Aujeszkyûs disease virus or Suid herpesvirus 1 belongs to the alphavirus subfamily of the family *Herpesviridae*. It is the causative agent of Pseudorabies (Aujeszky's Disease), which is responsible for considerable economic losses in the swine industry worldwide. The pig is the only natural host of the PR virus, which accounts for its ability to be subclinical and latent infection (Kluge et al., 1999). To reduce the economic losses, vaccination is widely practiced in many countries. However, it is well known that a vaccinated pig can still be infected and shed virulent PR virus. Consequently,vaccination program as such will not lead to eradication this virus, but the vaccine does decrease the likelihood of wild virus circulation to the commercial pig industry. For control of PR virus in such countries, an urgent need exists for a serological assay to distinguish infected from vaccinated pigs (Van Oirschot et al., 1988, Mellencamp et al., 1989, Eloit et al., 1989, Morenkov et al.,1997, Gut et al., 1999). The glycoprotein gE (gE) of PR virus is expressed by all wild-type virus strains tested so far (Van Oirschot, 1989; Morenkov et al., 1997), where as some vaccine virus strains genetically engineered deletion mutants lack gE (Hampl et al., 1984, Mettenleiter et al., 1985, Gielkens et al., 1989). In Thailand, PR virus eradication program has been implemented by using vaccines based on gE

negative (gE$^-$) PR virus strains and corresponding testing for gE specific antibodies, which differentiate infected from vaccinated pig. Therefore, serological tests to detect antibodies against gE is crucial for the eradication of PR virus.

Glycoprotein E which is non-essential for virus replication in tissue culture can induce neutralizing and protective antibodies. The role of gE was defined. It was shown that gE in duces a long-lasting humoral immune response in infected swine (Van Oirschot et al.,1988, Van Oirschot, 1989). It was also found that infected animals respond differently to different epitopes of gE, and the epitope-specific antibody response varies considerably in individual swine (Jacobs et al., 1994). Five of the epitopes were located in the N-terminal part of the protein, which is considered to be the immunodominant region of gE (Fuchs et al., 1990; Jacobs et al., 1990). The N-terminal part of gE was presented at amino acids 33 to 108, where at least three different epitopes binding complement-dependent neutralizing antibodies are located (Fuch et al., 1990).The recombinant gE expressed in *E. coli*, amino acids 77-210, reacted specifically with serum from PRV- hyperimmunized pigs and from field PRV-infected pigs, but not with serum samples from specific pathogen free pigs or pigs inoculated with gE-deleted PRV vaccine (Ro et al., 1995). Also, the sera from pigs infected experimentally with PR virus reacted with the antigenic domain of glycoprotein E at amino acids 52 to 238 (Jacob et al., 1990). These data indicate that, although the recombinant gE protein is produced in *E. coli*, it still retains the antigenicity of the viral gE glycoprotein. The objective of this study was to produce a large quatity of functional gE protein by cloning and expression of an antigenic domain of glycoprotein gE of field stain (PR virus 396) in *E. coli* system for produce polyclonal antibodies and application for serological diagnostic.

MATERIALS AND METHODS

Virus and Plasmids

The wild type PR virus 396 stain which was isolated from Kampangsean Veterinary Diagnostic Laboratory, Faculty of Veterinary Medicine, Kasetsart University, Kumphaengsean campus, Nakorn Pathom and the gE-PR virus vaccine are cultured with SK6 continous cell lines. The swine fever virus (SFV) and porcine reproductive and respiratory syndrome-US strain (PRRSV-US) are used for investigation of immunological cross reaction. The *E. coli* TOP 10 (INVITROGEN) and M15 (QIAGEN) are used as host for gene cloning and expression, respectively. The pCR2.1 TOPO (INVITROGEN) and pQE40 (QIAGEN) are used as vector for gene cloning and expression, respectively.

Gene construction

The wild type PR virus 396 strain was used for DNA extract by alkali lysis method and DNA amplification with PCR technique. The oligonucleotide primer sequences of antigenic domain of glycoprotein gE of pseudorabies virus were synthesized based on the nucleotides sequence which been reported in Genebank (Petrovskis et al., 1986), encoding 37 amino acids (54-90 aâ fragment) as follow; primer-1: > **GGA TCC** GAC GAT GAC CTC GAC GGC GAC C<3' and primer-2: 5'> **GGA TCC** TAG GAC ACG TTC ACC AGA TGG GCC GG <3'. The PCR condition was pre-denaturation at 96°C for 3 minutes, subsequently performed with 35 cycles of PCR amplification; denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute and extension at 72°C for 30 seconds, and final extension for an additional at 72°C for 7 minutes. The PCR product was visualized under UV illumination, and the products size was 122 bp. The PCR product was cloned to pCR2.1 TOPO cloning vector as recommended. Positive clone was isolated for plasmid DNA and sequenced, respectively. The *Bam* HI was used for cutting gE gene and ligated to *Bgl* II digested pQE40 (Sambrook et al.,1989). The oligonucleotide fragment encoding for the antigenic domain of gE was fused to T5 promoter and *lac* operator and in the same reading frame as a fusion protein mouse dihydrofolate reductase (mouse DHFR) and transformed to M15 (Ausubel et al., 1995).

Recombinant PR virus-gE (rPR virus-gE) Expression

The pQE40-gE M15 was cultured in LB medium containing both 100 µg/ml ampicillin and 25 µg/ml kanamycin at 37°C in vigorous incubator for overnight. The culture was subcultured to both antibiotic containing LB medium at 1:50 dilution of similar condition until the OD 600 between 0.5-0.7 was achieved. The solution was aliquoted for 1 ml as negative control. Then, the subculture was induced with 1 mM IPTG and cultured for 4-6 hours thereafter. The gene expression was analyzed with 10% SDS-PAGE.

The rPR virus-gE protein preparation

The IPTG induced pQE40 M15 was centrifuged at 5,500 rpm for 5 minutes. The protein partial purification was done according to the method described previously (Lertwatcharasarakul et al., 2003). Briefly, the cell pellet was resuspended in 2M urea and incubated at room temperature for 30 minutes. The suspension was sonicated on ice for 30 sec. 4 times and centrifuged at 10,000xg at 4°C for 15 minute. The pellet was resuspended in 4M urea and incubated at room temperature for 30 minutes before centrifugation. The pellet was resuspended in 1% SDS. The entire supernatants and suspension were kept at-20°C until analysis by SDS-PAGE.

Animal immunization and serological analysis

The 4M urea suspension was separated through SDS-page and staining with 0.3M $CuCl_2$ and purified according to the method previously described (Lertwatcharasarakul et al., 2003). The rPR virus-gE pellet was mixed with complete Freund' adjuvant, injected to Balb/C mouse. The mouse was boosted with incomplete Freund' adjuvant mixed protein 2-3 weeks after. The serum was collected before and after protein injection and absorbed non-specific protein (Lertwatcharasarakul et al., 2003) before screening test with dot blotting technique. The high titer serum was used for differentiation of wild type and vaccine (gE^-) PR virus infected cell line by immuno-peroxidase assay and immuno-fluorescence assay. The CSFV and PRRSV-US infected cell were used for detection of cross reaction.

RESULTS AND DISCUSSION

The wild type PR virus 396 was isolated from infected pig and extracted DNA. The oligonucleotide primers were used amplified DNA by PCR. The PCR product is 122 bp in size (Figure1). The rPR virus-gE gene was cloned in pCR2.1 TOPO and transformed to TOP 10 *E. coli.* The PCR positive clone was corrected for sequencing assay and compared with gE gene in Gene Bank (Petrovskis et al., 1986). The result was a 100% precision. The *Bam* HI digested fragment gene was ligated with *Bgl* II digested pQE40 and transformed to M15 *E. coli.* The PCR positive clone was sequenced. The IPTG was used for induction of protein expression. The result showed that recombinant protein was 26.5 kDa in size (Figure 2) when compared with protein marker (Promega). From the investigation of recombinant protein characteristic, the result showed that the rPR virus-gE proteins were accumulated in inclusion bodies approximately 20% of total *E. coli* protein. For partial protein purification, the 2M urea with sonication can wash almost *E. coli* protein from rPR virus protein. The rPR virus-gE proteins were solubilized almost in 4M urea and showed a slightly rest of protein in 1% SDS solution (Figure 3)

After the mouse immunization, the normal mouse serum and mouse anti-gE serum were screening for antibody titer and non-specific back ground with dot blotting. The antibody titer was increased after third injection at 1:6400 with a slightly back ground at 1:800 dilutions. These results indicated that the recombinant protein expressed in *E. coli* system and denature condition still contained the antigenicity and the non-specific protein absorb solution can effectively decrease the non-specific back ground. The IPMA and IFA were used for investigated specificity and sensitivity of polyclonal antibodies. The IPMA and IFA have similar capability to differentiate wild type from vaccine PR virus infected cell line at 1:100 to 1:800 and 1:1,200 dilutions, respectively (Table 1).

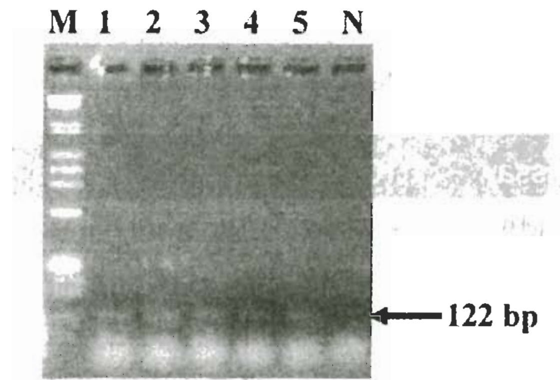

Figure 1. 2% agarose gel electrophoresis analysis of PCR assay of rPR virus-gE gene that showed the PCR products at 122 bp. M = DNA marker (λDNA *Hind* III *ΦX- Hae* III), lane 1-5 = positive clones, lane N = negative clone

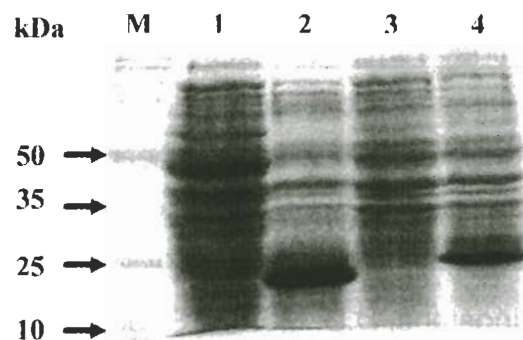

Figure 2. SDS-PAGE analysis of rPR virus –gE expression in *E. coli* M15. Lane M; Protein marker (Promega). Lane 1; *E. coli* M15 containing pQE40 without IPTG induction. Lane2; *E. coli* M15 containing pQE40 with IPTG induction. Lane 3; *E. coli* M15 containing rPR virus-gE without IPTG induction. Lane 4; *E. coli* M15 containing rPR virus with IPTG induction.

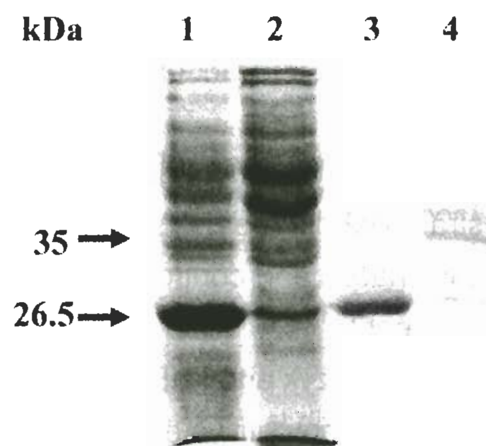

Figure 3. SDS-PAGE analysis of rPR virus-gE expression in *E. coli* M15. Determination of protein solubility with urea. Lane 1; *E. coli* M15 containing rPR virus-gE with 1mM IPTG induction. Lane 2; Supernatant from 2m urea and sonicated extraction. Lane 3; Supernatant from 4M urea extraction. Lane 4; 1% SDS suspension

Table 1. IPMA and IFA result of PR virus comparative with PR virus vaccine strain, CSFV and PRRSV-US, NT = Not test, + = Positive result, - = Negative result.

Virus/ 1' Ab dilutions	Assay	1:100	1:200	1:400	1:800	1:1,200
PR virus	IPMA	+	+	+	+	NT
	IFA	+	+	+	+	+
Vaccine PR virus	IPMA	-	NT	NT	NT	NT
	IFA	-	NT	NT	NT	NT
CSFV	IPMA	-	NT	NT	NT	NT
	IFA	-	NT	NT	NT	NT
PRRSV-US	IPMA	-	NT	NT	NT	NT
	IFA	-	NT	NT	NT	NT

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