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Production and diagnostic application of a purified, *E. coli*-expressed, serological specific chicken anemia virus antigen VP3

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Summary

The aim of this study was to evaluate the production of chicken anemia virus VP3 protein in different *E. coli* strains and to address the diagnostic application of purified *E. coli* expressed VP3 protein for the detection of CAV infection and the development of an ELISA kit. Three *E. coli* strains, BL21, BL21 codonplus RP and BL21 pLySs, each harboring a VP3 protein expressing plasmid, were investigated after induction to produce recombinant VP3 protein. After IPTG induction, VP3 protein was successfully expressed in all three *E. coli* strains. The BL21 pLySs strain gave the best performance in terms of protein productivity and growth profile. In addition, the optimal culture temperature and IPTG concentration were found to be 0.25 mM and 20℃, respectively. Using Ni-NTA-purified VP3 protein as an ELISA coating antigen, the purified VP3 was shown to be highly antigenic and able to discriminate sera from chickens infected with CAV from those that were uninfected during an evaluation of CAV infection serodiagnosis. A VP3-based ELISA demonstrated 100% (6/6 × 100%) specificity and sensitivities of 91.3% (21/23 × 100%) and 82.6% (19/23 × 100%) using cut-off values of the mean plus 2 S.D. and the mean plus 3 S.D., respectively.

Keywords: chicken anemia virus, VP3, recombinant protein, production, diagnosis

Introduction

Chicken anemia virus (CAV) is an important veterinary pathogen worldwide that infects young chickens. This virus was first isolated in Japan and has been identified as the major chicken anemia agent responsible for the induction of various clinical disease including anemia, aplasia of bone marrow and atrophy of thymus (Adair, 2000). The virus belongs to the genus Gyrovirus of the family Circoviridae and contains a circularized negative sense single-stranded DNA genome of about 2.3 kb, which consists of three open reading frames (ORFs) (Claessens et al., 1991; Kamada et al., 2006; Koch et al., 1995). The three ORFs respectively encode VP1 (51 kDa), VP2 (28 kDa) and VP3 (13 kDa), which partially overlap on the CAV genome. During CAV infection, the accumulation of VP2 and VP3 in host cell has been reported to occur within 12 hours post infection, while VP1 is detected only after 24 hours (Lucio et al., 1990). Epidemiological studies have shown that almost all new-born chicks are susceptible to CAV infection (Deng et al., 2009). Generally, young chicks less than two weeks old are very susceptible to CAV infection through the vertical transmission. In Taiwan, a mortality rate as high as 55% and a morbidity rate of 80% have been reported when chicks are infected with CAV (Hsu et al., 2002). Therefore, the monitoring of CAV infection is a critical step when controlling CAV infection.

At present, there are several conventional methods that can be used to diagnose the CAV pathogen (McNulty et al., 1989 & 1990; Noteborn et al., 1991 & 1998; Brentano et al., 2005; Iwata et al., 1998). Enzyme-linked immunosorbent assay (ELISA) is a popular assay for detecting CAV infection and screens for CAV-specific antibodies in the sera of the chickens (McNulty et al., 1990; Noteborn et al., 1991;

Iwata et al., 1998). However, the propagation of CAV in cell culture or chicks is not convenient, is time-consuming and has a low yield. Moreover, virion purification is also a tedious and cost-ineffective process. Therefore, DNA recombinant technology has been chosen as a better way to produce CAV viral protein for use as an ELISA antigen. Previously several expression systems, including *E. coli*, baculovirus-insect cells and plant cells, have been exploited to express CAV viral proteins (Noteborn et al., 1991; Iwata et al., 1998; Lacorte et al., 2007; Wang et al., 2007; Pallister et al., 1994; Lee et al., 2009). Among these, the *E. coli* expression system is easier to operate and more economic to apply for viral protein production. In terms of cytotoxicity, it is still difficult to express full-length VP1 protein in *E. coli* because this protein is rich in highly charged amino acids (Pallister et al., 1994; Lee et al., 2009). Therefore, VP2 and VP3 protein expression in *E. coli* has been employed in a number of previous studies. Among these two non-structural proteins, the lower molecular weight of VP3 at 13 kDa makes it more suitable for expression in *E. coli* than the VP2 protein at 26 kDa (Lacorte et al., 2007; Pallister et al., 1994; Nogueira-Dantas et al., 2007). However, recombinant VP3 protein has rarely been explored for protein production in *E. coli* and neither has this protein been exploited to any great extent for use in ELISA systems that examine chicken sera.

In this study, we examine a number of different recombinant *E. coli* strains harboring a CAV VP3 protein expression cassette that can be used to produce recombinant VP3 protein. This was done with the aim of systematically assessing protein productivity under various operational parameters and treatments. Moreover, the purified *E. coli*-expressed VP3 was then used as coating antigen in an ELISA assay and gave good performance in terms of antigenicity and specificity when detecting VP3 antibodies.

Materials and methods

Construction of VP3 expression vectors

The VP3 cDNA was amplified from our previous plasmid pGEX-1-6P-VP2 by PCR (Huang et al., 2010). Standard recombinant DNA methods were used and followed the approaches used in previous studies for plasmid construction（Sambrook et al., 1989）. To introduce an *Nde* I and *Xho* I cleavage site into the 5’ and the 3’end of the CAV VP3 gene, respectively, one specifically designed PCR primer set, CH103 (5’-ggaattccatatgaacgctctccaagaag-3’) and CH54 (5’- ccgctcgagcagtcttatacaccttcttg-3’) was used to perform PCR using the plasmid pGEX-6P-1-VP2 as template DNA. The resultant PCR product was cloned into plasmid pVI127 (modified from pET21b by adding the TAT sequences from HIV at the 5' terminus of the multi-cloning site and introducing His-tags at the 5’ and 3’ ends of the multi-cloning site. The resultant construct, pVI127-VP3, was analyzed by restriction enzyme digestion and sequencing before it was used for further protein expression.

VP3 protein expression and purification

Three recombinant *E. coli* strains harboring the VI127-VP3 plasmid were used to express VP3 protein under induction by 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 hours. The recombinant strains were cultured in LB medium and the culture condition used were as described in previous studies (Lee at al., 2009). For purification of the recombinant VP3 protein under denaturing conditions, the detailed procedures and the buffer solutions were the same as described in our previously work (Lee at al., 2009). The VP3 proteins were analyzed by 15% SDS–PAGE and visualized by staining with Coomassie blue.

Quantification of the E. coli-expressed VP3 proteins

The *E. coli* cell pellets expressing VP3 protein were collected by centrifugation at 12000xg for 10 mins and dissolved in PBS. The dissolved volume was determined according to the OD_{280}. After disrupting the cells by sonication using a 20% pulsed activity cycle (Vibra cell, Sonics & Materials, Inc.), the soluble protein extract and insoluble protein pellet was separated by centrifugation at 20000xg for 30 mins. Both soluble and insoluble protein were resolved on 15% SDS-PAGE, and then transferred to PVDF membrane. Western blot analysis was performed using an antibody targeting the His-tags. The banded protein intensity was analyzed by ImageQuant TL software (GE Healthcare) using purified VP3 as standard for protein content calibration. The concentration of recombinant VP3 proteins was measured by BCA protein assay (Lee at al., 2009).

Recombinant VP3 protein based enzyne-linked immunosorbent assay

ELISA plates (Nunc) were coated with 100µl volume of 10µg/ml purified recombinant VP3 protein in coating buffer (0.35 mM $NaHCO_3$, 0.15 mM Na_2CO_3, 0.1% NaN_3, pH 9.6). After coating at 4℃ for 16 h, blocking was carried out with 200µl of blocking buffer (5% skim milk in PBS) at 37℃ for 1 h. Each well was then washed thoroughly with PBS containing 0.1% Tween-20 (PBST) three times. After washing, each well received 100 µl of 1000x diluted serum samples in PBS and the plates were incubated at 37℃ for 1 h. Subsequently, the plates were washed with PBST five times, each for 5 min; the plates were then treated with horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Jackson) diluted 1: 4000 in PBS at 37℃ for 45 min. Following three similar washes, 100 µl of freshly prepared chromogen/substrate solution (ABTS single solution, Zymed) was added into each

well and the plate was incubated at room temperature for 20 min. The optical density of each well was read at 405 nm using a microplate reader (Thermo). Each sample was repeated three times.

Matrix-Assisted Laser Desorptiony Ionization Mass Spectrometric (MALDI-MS)

Protein gel bands were extracted for tryptic mapping by MALDI-MS in a similar manner to our previous study (Lee at al., 2009). Individual protein bands were respectively excised, destained, washed, and digested with modified trypsin (Nnheim); the resulting peptides were extracted with acetonitrile. After vacuum drying, each sample was redissolved in 10μl reaction buffer containing 1% trifluoroacetic acid and 50% acetonitrile. A portion (0.5 ml) of this sample solution was loaded into the MALDI-MS sample plate together with 0.5 ml of matrix solution (2, 5-dihydroxybenzoic acid). MALDI-MS measurements were obtained using a delayed extraction time-of-flight mass spectrometer (Voyager DE PRO, Applied Biosystems) operated in reflector mode.

Results

Construction of the expression plasmid

In the CAV DNA genome, the three open reading frames (ORFs) partially overlap (Claessens et al., 1991; Kamada et al., 2006; Koch et al., 1995). As ORF1 within the VP2 gene, the full-length VP3 gene, which consists of 323 nucleotides, is present in this ORF as illustrated in Fig. 1A. To express the VP3 protein of CAV, the VP3 cDNA was created by PCR using the VP2 cDNA of CAV as the template DNA. By PCR with the specifically designed primers, the VP3 gene was amplified and cloned into pVI127 using the *NdeI* and *XhoI* restriction sites, which created a protein with

in-frame His-Tags. The resultant construction, pVI127-VP3, is shown in Fig. 1B. This plasmid was then transformed into the various *E. coli* strains for further testing of protein expression.

Expression and production of VP3 protein using different recombinant *E. coli* strains

To examine the expression of VP3 protein in *E. coli*, three *E. coli* strains, BL21, BL21 codonplus RP and BL21 pLySs, each harboring the pVI127-VP3 plasmid, were induced with 1 mM IPTG at 37℃ for 4 hours. As illustrated in Fig. 2B, the protein expression patterns of the bacterial total extract of the three *E. coli* strains were examined by SDS-PAGE and Western-blot assay. The results demonstrated that the different *E. coli* strains used are able to successfully express the recombinant VP3 under IPTG induction. The total expressed VP3 protein, including soluble and insoluble portions, of BL21 pLySs was found to be greater than that produced by BL21 or BL21 codonplus RP (Fig. 2C). In terms of soluble protein, the VP3 protein productions of the three different strains were almost the same (0.037, 0.038, 0.055 mg/mL by BL21, BL21 codonplus RP and BL21 pLyss , respectively). In contrast, in terms of insoluble VP3 protein, the protein productivity of the BL21 pLySs strain was found to be much higher (0.37 mg/mL) than the BL21 (0.01 mg/mL) or codonplus RP strains (0.01 mg/mL), respectively. In terms of the growth profiles of the *E. coli* strains, there were significant differences in the effect of IPTG on the growth of three *E. coli* strains in terms of the OD value at 600 nm (OD_{600}) (Fig 2A). The OD_{600} growth of BL21 pLySs was higher than that of either BL21 or BL21 codonplus RP (Fig. 2A).

Effect of IPTG concentration on the production of VP3 protein in *E. coli*

To examine the effect of IPTG concentration on protein productivity, various concentrations of IPTG were used for induction. Increasing the concentration of IPTG (from 0.05 to 0.25 mM) with *E.coli* BL21 pLySs obviously improved the production of insoluble VP3 protein from 0.27 to 0.86 mg/mL (Fig. 3A, 3B). However, when a level higher than 0.25 mM IPTG was used, the highest productivity reached was only about 0.5mg/mL. Therefore there was no significant improvement in protein productivity at these high levels of IPTG. In contrast to the situation in terms of insoluble VP3 protein, increasing the concentration of IPTG (from 0.05 to 1 mM) had no effect on the productivity of soluble VP3 protein with *E.coli* BL21 pLySs. Among the above, therefore, 0.25 mM IPTG was determined to be the optimal concentration for VP3 protein production using *E.coli* BL21 pLySs strain.

Effect of temperature on the production of VP3 protein in *E. coli*

To evaluate the effect of temperature on production of VP3 protein, the recombinant BL21 pLySs strain was used. Figure 4A shows the various levels of productivity for soluble and insoluble VP3 protein at three different temperatures, 20℃, 30℃ and 40℃. Under IPTG induction, both insoluble and soluble VP3 increased with declining temperature. Moreover, there were significantly differences in the growth profiles of the *E. coli* strains across three different temperatures in terms of OD value at 600 nm (OD_{600}), which are illustrated in Fig. 4C. The growth (OD_{600}) of *E. coli* BL21 pLySs at 30℃ was higher than at 20℃ or 40℃ at 4 hrs post-induction. After 8 hr post-induction, it was no obviously difference in OD_{600} over the three temperatures.

Purificationt, characterization and antigenicity of *E. coli*-expressed VP3 protein

To purify the *E. coli*-expressed VP3 protein, Ni-NTA affinity resin was used.

After affinity chromatography purification, the presence of purified denatured VP3 protein was confirmed (Fig. 5A). The purity of the VP3 protein was close to homogenicity in the elution fraction, as shown by SDS-PAGE (Fig 5A). In addition, the His-tag fused VP3 protein was recognized by anti-His-tag antibody using Western-blot analysis (Fig. 5A). This confirms that the purification of His-tag fused VP3 protein is feasible by Ni-NTA resin. Moreover, after examining the purified VP3 protein by mass spectrometry, six peptides from VP3 were identified from the trypsin digest. These demonstrated a good alignment with a high score. The longest peptide fragment, RSCDPSEYRVSELKENLITTTPSRPR, consisted of 26 amino acid residues (Fig. 5B). The identity of this peptide sequence completely matched that of CAV VP3 protein. All together the MS results provided 52% (64/121 amino acids) exact match coverage of the previous reported amino acid sequences of VP3 (Accession No. M55918.1 for VP3 [22]). Therefore, we concluded that the *E. coli* expressed VP3 protein had indeed originated from CAV. In addition, the purified protein also reacted with CAV positive serum specifically (Fig. 5A) and there was no cross reaction with CAV negative serum (data not shown). Taken together these results demonstrated that the purified *E. coli*-expressed VP3 protein will be able to act as a potential antigen candidate with high antigenicity when used for the development of a detection kit for CAV infection.

Application of VP3 protein based ELISA for diagnosis of CAV infection

To apply the *E. coli*-expressed VP3 as coating antigen for the diagnosis of CAV infection, an CAV VP3-based indirect ELISA was set up to evaluate the possibilities in terms of detection kit development. As shown as in Fig. 6, five CAV-negative and 23 CAV-positive specific chicken sera, which were collected from an experimental

farm, showed different levels of reactivity against the VP3 protein as measured by the OD value at 405 nm (OD_{405}). Purified VP3 protein demonstrated poor reactivity against CAV-negative sera. In contrast, purified VP3 protein demonstrated a high antigenicity and there was a significant difference in the OD values between the CAV negative and positive sera ($p<0.01$). This indicated the VP3 protein is highly antigenic and can discriminate chicken sera that have been CAV infected from those that have not been infected. Additionally, the individual OD_{405} values of the six CAV-negative chicken sera against VP3, as shown in Table 1, were averaged to define a positive threshold. The OD_{405} values obtained from the CAV-positive sera were all higher than this cut-off value. In addition, when the positive/negative cut-off values were determined as the mean plus 2 standard deviations (mean + 2 S.D.) or plus 3 standard deviations (mean + 3 S.D.), all VP3-based ELISAs demonstrated 100% (6/6 × 100%) specificity. The sensitivities of the VP3-based ELISA were 91.3% (21/23 × 100%) and 82.6% (19/23 × 100%) when the cut-off values were set at mean plus 2 S.D. and plus 3 S.D., respectively.

Discussion

The VP3 protein of CAV was successfully produced by the three *E. coli* strains used in this study. The production of VP3 protein has been reported for various eukaryotic and prokaryotic systems (Noteborn et al., 1998; Iwata et al., 1998; Nogueira-Dantas et al., 2007) and the reason for this is that the VP3 protein has important serodiagnostic applications. The cost of antigen production is related to the source of the antigen. Using recombinant antigen for serodiagnosis has several advantages, such as lower costs, ease of production and the fact that the antigenicity of recombinant proteins is simpler to characterize. Indeed, the final factor is a critical

consideration when developing a diagnostic kit to detect a pathogenic infection. The plant expressing system was found to have a number of disadvantages when producing CAV VP3 protein, such as the tedious procedure when constructing a transfomant, the very low production yield and the uncharacterized nature of the protein's antigenicity (Lacorte et al., 2007). When an insect cell-baculovirus system was used, the serum supplemented culture medium was costly and the whole system was not easy to maintain (Noteborn et al., 1998; Iwata et al., 1998). Therefore, a prokaryotic expressing system would seem to be more suitable as an expression system for the production of CAV VP3 protein. A previous study showed the VP3 of CAV, when expressed in *E. coli*, is able to produce 200μg/100 ml of protein (Nogueira-Dantas et al., 2007). Nonetheless, up to the present, how to obtain the optimal productivity of VP3 in any of the above systems has not been addressed. To the best of our knowledge, this is first report to evaluate the production of VP3 protein in different *E. coli* strains and to determine the optimal parameters for culturing these three *E. coli* strains to produce VP3. In this study, the three different recombinant *E. coli* strains used for protein production were BL21, BL21 codonplus RP and BL21 pLySs, each harboring pVI127-VP3. BL21 pLySs was found to give the best performance in terms of both protein productivity and growth profile (Fig 2A, 2B and 2C). The VP3 protein of CAV is a nuclear protein with DNA binding activity (Tavassoli et al., 2005). Previous studies have been demonstrated that expression of a DNA binding protein might be harmful to the growth of the host cell [17 24, 25]. In addition to this potential problem, expression of VP3 protein at high levels may result in a metabolic burden on the host that could influence maximal growth rate or protein productivity, especially this may be true for the BL21 and BL21 codonplus RP strains (Pallister et al., 1994; et al., Saïda et al., 2006; Miller et al., 1989). In addition to this potential problem, expression of VP3 protein at high levels may result in a metabolic

burden on the host that could influence maximal growth rate or protein productivity, especially this may be true for the BL21 and BL21 codonplus RP strains (Kurland et al., 1996). The present study demonstrates that, notwithstanding the above potential problems, BL21 pLySs strain has good potential when used to produce large amounts of recombinant VP3. This is possibly because growth rate and protein productivity characteristics of this strain are more suitable than those of the other strains.

IPTG is used to induce over-expression when an IPTG-inducible promoter is present. However, the cost of inducer, contamination of the end-product and the toxicity of the inducer have been suggested as problems that may limit its use in terms of protein production, the quality of the final product and an efficient recovery process (Figge et al., 1988; Baneyx et al.,1999). Thus, the IPTG concentration used herein is important when addressing protein over-expression in *E. coli*. The other parameter that may affect protein production is temperature, which can influence protein productivity and protein solubility. A previous study has been shown that lowering the bacterial growth temperature decreases periplasmic aggregation and increases the yield of soluble protein (Baneyx et al.,1999) and our results in this study agreed with this finding. Under IPTG induction, both insoluble and soluble VP3 protein production by BL21 pLySs increased with declining temperature. Combining the results of protein productivity and growth rate, a culture temperature between 20℃ and 30℃ would seem to be optimal for VP3 protein production (Fig. 4B, 4C). Using the optimized conditions for VP3 production in terms of different bacterial strains, temperature and induction conditions, the productivity of VP3 was estimated to be 1.99mg/mL approximately (data not shown) for BL21 pLySs, which is a much higher productivity than published in previous studies (Nogueira-Dantas et al., 2007).

The His-tag fusion system is a versatile and popular method for expression, purification and detection of fusion proteins. In this study, we constructed

pVI127-VP3 with two His-tags at either end of the VP3 protein to allow recovery during downstream processing; our results indicated that it is feasible to purify the VP3 protein by Ni-NTA column in one step. Moreover, this process is easy to scale-up for commercial protein production in the future. Importantly, the purified *E.coli* expressed VP3 protein possesses discriminating immunorelevant epitopes and has high sensitivity when reacting with CAV specific antibodies. The VP3-based ELISA assay developed in this study was found to be a useful serodiagnostic kit for detection of CAV infection. In addition to using the full length VP3 protein for such kits, antigenic domains screening of the VP3 protein in the future will be helpful in improving the sensitivity of the indirect ELISA.

In summary, the full-length CAV VP3 gene was cloned and the production parameters of the VP3 protein in *E.coli* were investigated. The purified *E.coli* -expressed VP3 protein described here has the potential to become a valuable candidate as an ELISA coating antigen for developing CAV antibodies detection kits. In this context, the *E. coli*-expressed VP3-based ELISA established here shows high specificity and sensitivity. These results provide useful information that will help the large-scale production of recombinant VP3 protein for routine serodiagnosis, for epidemiological investigations of CAV infection and will help with assessing the immunization efficacy after vaccination of farm-bred young chickens.

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Legends

Figure 1. Construction of recombinant CAV VP3 gene in a *E. coli* expression system (A). A 323-bp of VP3 gene was amplified from VP2 gene; and cloned into *E. coli* expression vector pVI127 under control of T7 promoter (B). The primer set used for amplification of VP3 gene was illustrated by arrows. Two six-His tag fused to the N- and C-terminus of VP3 protein, respectively, was shown as hatched box.

Figure 2. (A) Growth profiles of recombinant *E. coli* BL21 (DE3), BL21 (DE3) codonplus RP and BL21 (DE3) pLyss, respectively, harboring pVI127-TAT-VP3 in LB medium during post-induction by adding of IPTG. (B) Expression of recombinant VP3 in three different recombinant *E. coli* strains was analyzed by SDS-PAGE and Western blot, respectively, during post-induction by adding of IPTG. (C) Production yield of recombinant VP3 in three different recombinant *E. coli* strains.

Figure 3. Effect of used IPTG concentration on the expression level of total VP3 protein. (A) SDS-PAGE and Western Blot were performed for analysis of recombinant VP3 expression under different concentration of IPTG induction. (B) The relative quantity of produced soluble and insoluble VP3 protein was respectively

determined at different IPTG concentration used for induction. The band-intensity was analyzed by AlphaDigiDocRT software.

Figure 4. Effect of the cultivation temperature on expression of recombinant VP3 protein in soluble form. (A) Western Blot analysis of expressed soluble and insoluble protein at various culture temperatures. All samples were analyzed under the same amount of cells loading; S, Soluble fraction; In, insoluble fraction. (B) The solubility of VP3 protein at different cultivation temperature was calculated as the ratio of band-intensity of soluble VP3 protein to total VP3 protein. The band-intensity was analyzed by AlphaDigiDocRT software. (C) Growth profiles of recombinant *E. coli* BL21 (DE3) pLyss harboring pVI127- VP3 in LB medium during post-induction by adding of IPTG at various culture temperatures.

Figure 5. Purification of *E. coli* expressed recombinant VP3 by Ni-NTA resin. (A) The purity of recombinant VP3 was determined by SDS-PAGE and Western Blot analysis. The antibodies against His-tag (middle panel) and CAV (right panel) were used respectively. (B) Amino acid sequence of the VP3 protein determined by MALDI-TOF. The red letters represent the actural amino acid sequence matched.

Figure 6. (A) Reactivity of chicken serum with recombinant VP3 protein determined by ELISA. Five CAV-negatives and 23 CAV-positive chicken serum were respectively used to react with VP3-based ELISA assay. The reactivity was determined in terms of obtained OD value at 405 nm (OD405). (B) Determination of cut-off value to reactivity of ELISA on CAV-negative chicken serum. Sera no.1-5 was obtained from SPF chickens in the experimentally farm. These sera were all identified as negative using commercial ELISA kit purchased from IDEXX laboratory Inc. Serum no.6 was negative serum obtained from IDEXX commercial kit. S.D., standard deviations.

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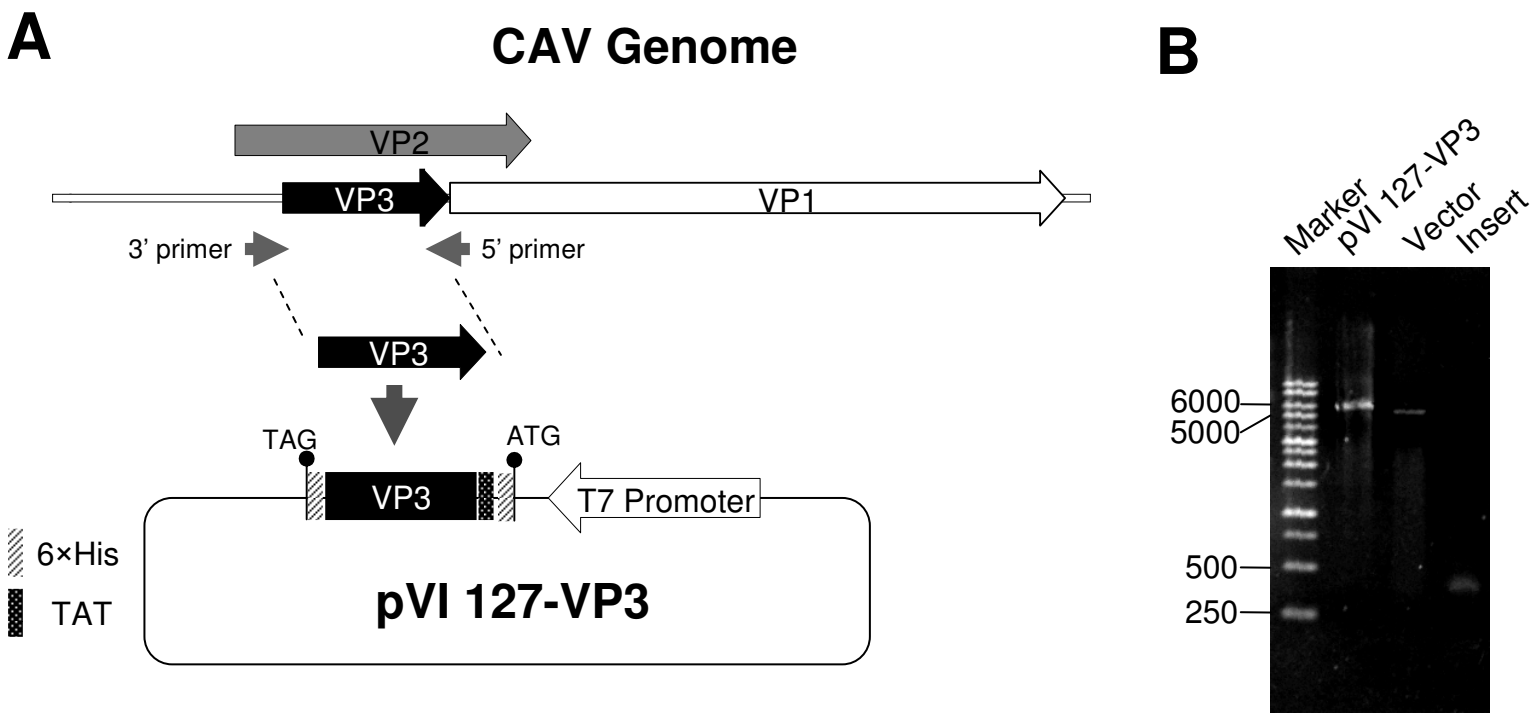


Figure 1. Construction of recombinant CAV VP3 gene in a *E. coli* expression system (A). A 323-bp of VP3 gene was amplified from VP2 gene; and cloned into *E. coli* expression vector pVI127 under control of T7 promoter (B). The primer set used for amplification of VP3 gene was illustrated by arrows. Two six-His tag fused to the N- and C-terminus of VP3 protein, respectively, was shown as hatched box.

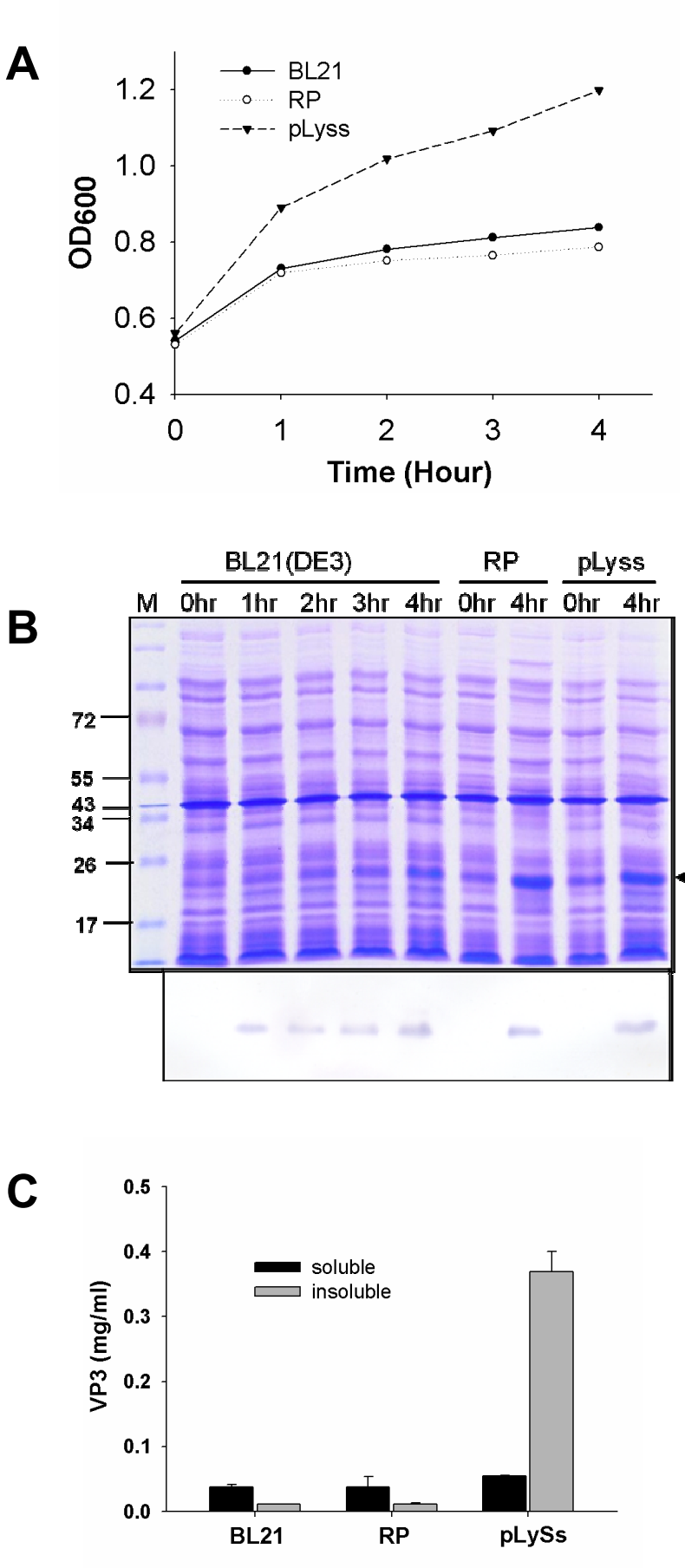

A
BL21
RP
pLyss
OD600
1.2
1.0
0.8
0.6
0.4
0
1
2
3
4
Time (Hour)
B
BL21(DE3)
RP
pLyss
M
0hr
1hr
2hr
3hr
4hr
0hr
4hr
0hr
4hr
72
55
43
34
26
17
C
VP3 (mg/ml)
0.5
0.4
0.3
0.2
0.1
0.0
soluble
insoluble
BL21
RP
pLySs

Figure 2. (A) Growth profiles of recombinant *E. coli* BL21 (DE3), BL21 (DE3) codonplus RP and BL21 (DE3) pLyss, respectively, harboring pVI127-TAT-VP3 in LB medium during post-induction by adding of IPTG. (B) Expression of recombinant VP3 in three different recombinant *E. coli* strains was analyzed by SDS-PAGE and Western blot, respectively, during post-induction by adding of IPTG. (C) Production yield of recombinant VP3 in three different recombinant *E. coli* strains.

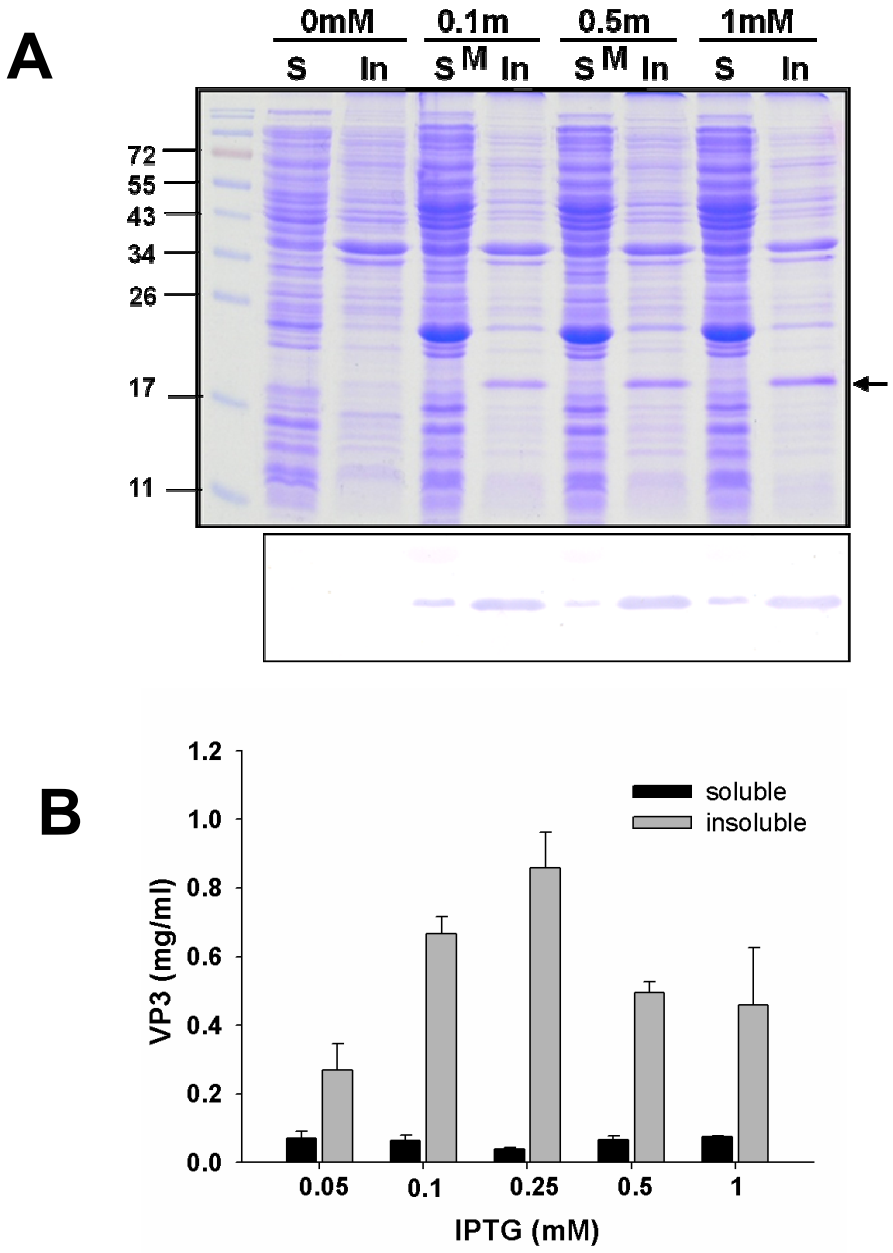
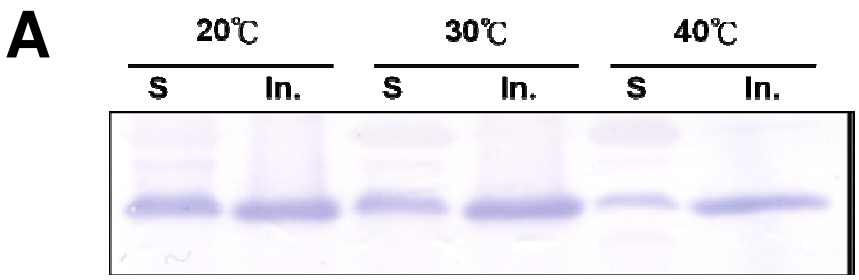
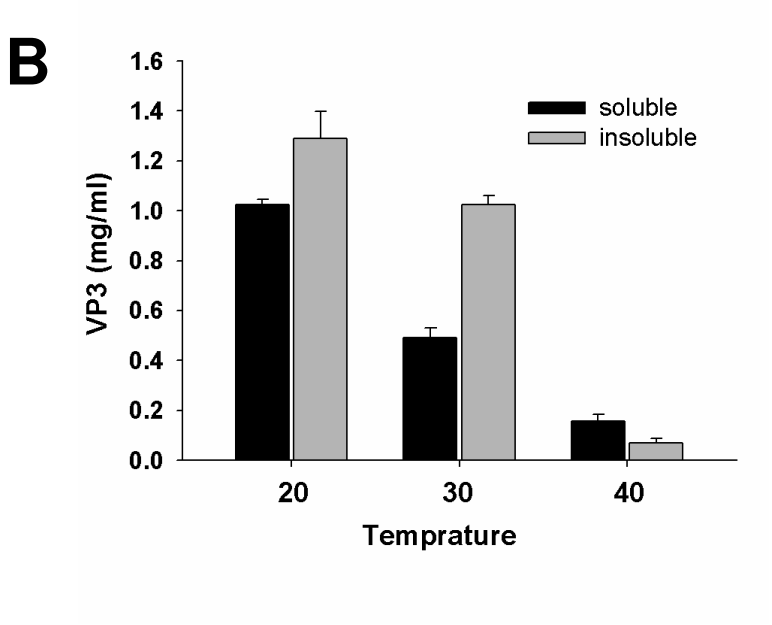


Figure 3. Effect of used IPTG concentration on the expression level of total VP3 protein. (A) SDS-PAGE and Western Blot were performed for analysis of recombinant VP3 expression under different concentration of IPTG induction. (B) The relative quantity of produced soluble and insoluble VP3 protein was respectively determined at different IPTG concentration used for induction. The band-intensity was analyzed by AlphaDigiDocRT software.


A
20℃
30℃
40℃
S
In.
S
In.
S
In.


B
VP3 (mg/ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
soluble
insoluble
20
30
40
Temprature

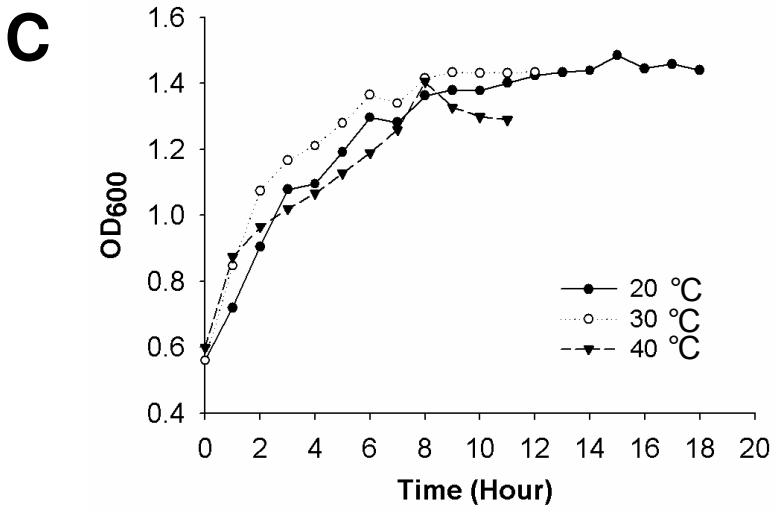

C
OD600
1.6
1.4
1.2
1.0
0.8
0.6
0.4
20 ℃
30 ℃
40 ℃
0
2
4
6
8
10
12
14
16
18
20
Time (Hour)

Figure 4. Effect of the cultivation temperature on expression of recombinant VP3 protein in soluble form. (A) Western Blot analysis of expressed soluble and insoluble protein at various culture temperatures. All samples were analyzed under the same amount of cells loading; S, Soluble fraction; In, insoluble fraction. (B) The solubility of VP3 protein at different cultivation temperature was calculated as the ratio of band-intensity of soluble VP3 protein to total VP3 protein. The band-intensity was analyzed by AlphaDigiDocRT software. (C) Growth profiles of recombinant *E. coli* BL21 (DE3) pLyss harboring pVI127- VP3 in LB medium during post-induction by adding of IPTG at various culture temperatures.

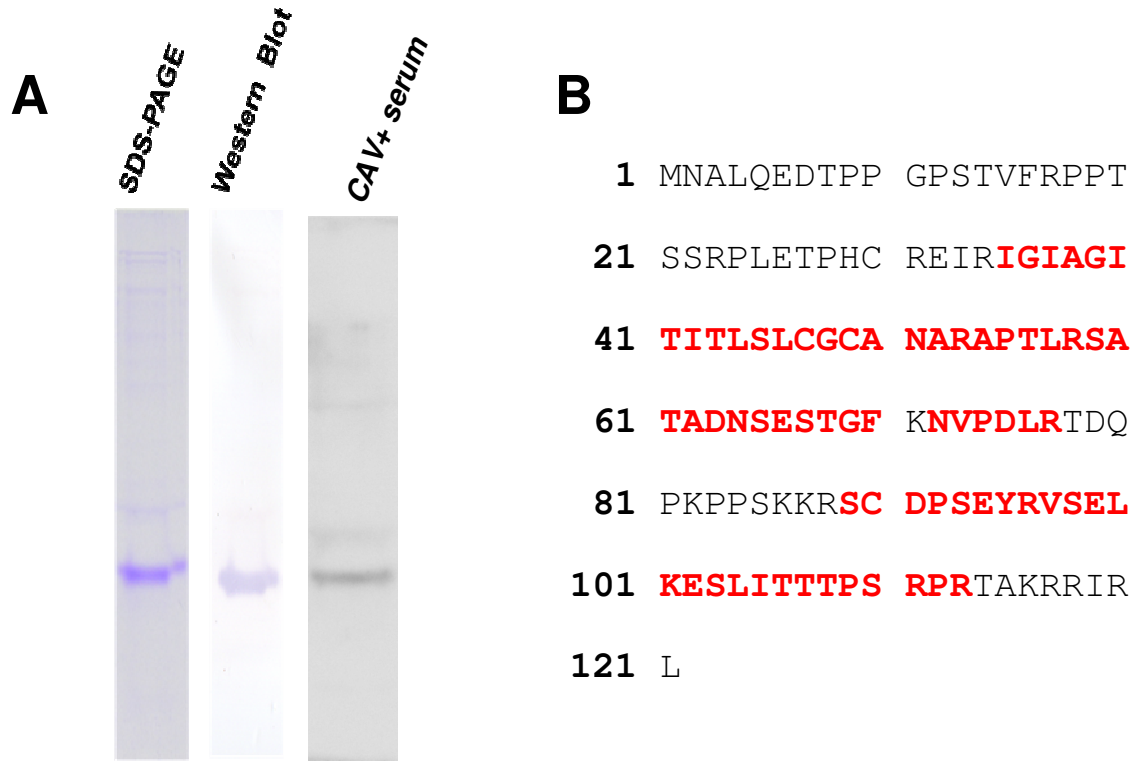
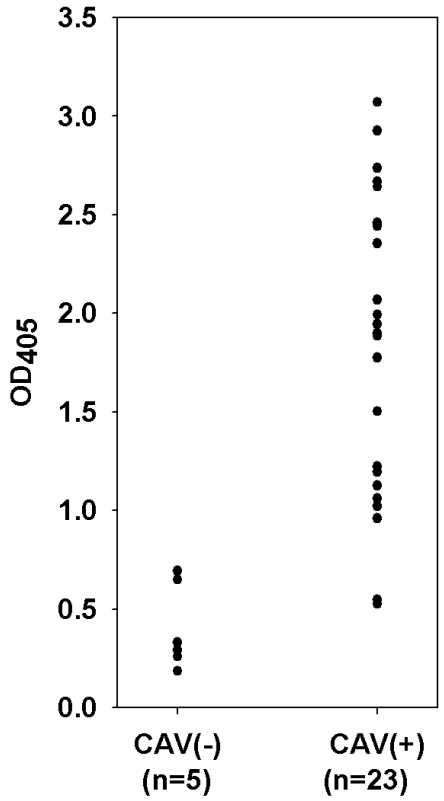


Figure 5. Purification of *E. coli* expressed recombinant VP3 by Ni-NTA resin. (A) The purity of recombinant VP3 was determined by SDS-PAGE and Western Blot analysis. The antibodies against His-tag (middle panel) and CAV (right panel) were used respectively. (B) Amino acid sequence of the VP3 protein determined by MALDI-TOF. The red letters represent the actural amino acid sequence matched.

A



B

Serum no.	OD_{405}
1	0.18589
2	0.25894
3	0.693734
4	0.330074
5	0.291546
6	0.649581
Mean	0.401627
S.D.	0.214917
Mean +2 S.D.	0.831462
Mean +3 S.D.	1.04638

Figure 6. (A) Reactivity of chicken serum with recombinant VP3 protein determined by ELISA. Five CAV-negatives and 23 CAV-positive chicken serum were respectively used to react with VP3-based ELISA assay. The reactivity was determined in terms of obtained OD value at 405 nm (OD405). (B) Determination of cut-off value to reactivity of ELISA on CAV-negative chicken serum. Sera no.1-5 was obtained from SPF chickens in the experimentally farm. These sera were all identified as negative using commercial ELISA kit purchased from IDEXX laboratory Inc. Serum no.6 was negative serum obtained from IDEXX commercial kit. S.D., standard deviations.