Ethiop. J. Agric. Sci. 24:69-82 (2014)

Expression and Purification of Coat Protein of Citrus Tristeza Virus

Surafel Shibru[1], Srimek Chowpongpang[2*], and Ampaiwan Paradornuwat[2]
[1]*Plant Biotechnology Research Program, Melkassa Agricultural Research Center, Ethiopian Institute of Agricultural Research, P.O. Box 436 Nazareth, Ethiopia*
[2]*Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkhen Campus, Bangkok, 10900 Thailand, E-mail agrsmc@ku.ac.th*

Abstract

Citrus tristeza virus (CTV) polyclonal antibodies produced either from the recombinant coat protein (CP) of CTV or extracted virus from midrib used for the detection of virus. Compared with intact virion procedure, the use of CP antigen resulted in highly specific polyclonal antibodies. CTV coat protein gene (CTV-*cp*) cloned in pQE30 vector and transformed to DH5α containing 666bp long from Thailand MK-50 isolate was amplified with a forward primer CTV-CP1 (5′ CAC CGA CGA AAC AAA GAA ATT GAA GAA CA 3′) and a reverse primer CTV-CP2 (5′ TCA ACG TGT GTT AAA TTT CCC AAG C 3′) and cloned into TOPO vector and transformed to TOP10 *E. coli* competent cell. Six colonies of TOP10 *E. coli* were selected and checked for the appropriate insertion of *cp* gene with PCR using T7F (5′ TAA TAC GAC TCA CTA TAG GG 3′) as forward primer and CTV-CP2 as reverse primer. Two colonies having appropriate insertion were selected for transformation into BLD21 star (DE3) expression *E. coli* cell and their recombinant protein expressions capacity and optimum length of time were studied after inducing with 1mM IPTG. One of the colonies was selected and used for mass production of recombinant protein and the produced protein was purified using Ni-NTA resin. The result indicated that the expression of recombinant CP was obtained only for cloned CTV-*cp* gene in TOPO vector within BLD21 star (DE3) *E. coli* cell and inducing protein for 4hours after addition of 1mM IPTG were given optimum amount of recombinant protein expression. The recombinant CTV-CP was highly bound to Ni-NTA resin and only eluted when washed with low pH buffer during the purification, and can be used for polyclonal antibodies production.

Keywords: CTV, *cp* gene, SDS-PAGE

Introduction

Citrus is one of the major fruit crop in Thailand and in present day production value of all citrus crops is far exceeding that of all deciduous tropical tree fruits (Paradornuwat 2004). *Citrus tristeza virus* (CTV) is one of the major threats for the production of Citrus worldwide. In Thailand, since October 1998, CTV is recognized as one of the most economically important disease of citrus in many production areas. Stem pitting, vein clearing, leaf mottling, leaf cupping, vein corking and symptomless are observed symptoms on infected plant (Paradornuwat *et al.* 1984; Paradornuwat 2004).

CTV is a phloem-limited and the longest known from plant virus particles in size approximately 2000-nm long, a single-stranded positive-sense RNA of about 20kb. The virons contain two capsid proteins (CPs), a 25 KDa CP (Sekiya *et al.* 1991; Pappu *et al.* 1992; Pappu *et al.* 1994) covering about 95% of the particle length and a small amount of a diverged 27 KDa coat protein (p27) (Karasev *et al.* 1995). The genome encodes 12 ORFs which potentially codes for at least 17 protein products (Mawassi *et al.* 1996).

Biological disease diagnosis using Mexican lime (*Citrus aurantifolia*) as an indicator plant is the common method to detect CTV infected plants, which usually induce typical vein clearing and cupping on leaves. Vein clearing can be detected in most isolates within 8 weeks in healthy vigorous Mexican limes grown under proper temperature conditions (Roistacher 2004). Indexing plant using this method can able to give a false negative result, since mild isolates are common in almost all the citrus growing areas (Albiach-Martí *et al.* 2000; Hilf *et al.* 2005; Roy *et al.* 2010), and their presence is frequently masked when they are present in mixed infections with severe isolates (Moreno *et al.* 2008). Moreover, there are also exceptional isolates which induce symptoms in sweet orange but not in Mexican lime (Harper *et al.* 2009).

Successfully developed the enzyme linked immunosorbent assay (ELISA) for the rapid detection kits of CTV was a momentous breakthrough (Bar-Joseph *et al.* 1979). Detection of CTV by ELISA opened the door for large scale indexing to determine the distribution of CTV within a grove area, region or country. This proved innovative and valuable for the rapid detection of CTV (Garnsey *et al.* 1993; Garnsey *et al.* 1993).

Polyclonal antibodies used for the detection of CTV, can be produced either using recombinant CTV-CP or purified viral particles of CTV infected midrib. In comparisons, the later was a laborious procedure and always contaminated with plant proteins, while applying the use of recombinant CP antigen resulted in highly specific polyclonal antibodies without cross reaction with plant protein. Using recombinant CP technique antigen, that can able to produce either polyclonal or monoclonal antibodies in injected animal, have been produced (Nurhadi *et al.* 2003; Sadeghan *et al.* 2013). Recently 8 Thailand CTV-CP isolates was successfully purified by one step RT-PCR from CTV infected midribs, the nucleotide and amino acid sequences of these isolates were compared to Florida T-36 (NC_001661), showed no deletion, insertion, and/or frame shift mutation (Paradornuwat *et al.* 2004). The expression and purification of CTV-*cp* gene of Thailand isolates were not studied before. Therefore, in this experiment Thai MK-50 isolate was used for the study purpose

Materials and Research Methodology

CTV- coat protein gene amplification and cloning

Thai MK-50 isolate of CTV-*cp* gene, which is harbored in pQE30 vector DH5α *Escherichia coli* competent cell (Paradornuwat *et al.* 2004), was used for the study purpose. Based on the nucleotide sequence of Thailand CTV MK-50 isolate a forward PCR primers CTV-CP1 (5′ CAC CGA CGA AAC AAA GAA ATT GAA GAA CA 3′) containing TOPO directional cloning site (the underline) and a reverse PCR primers CTV-CP2 (5′ TCA ACG TGT GTT AAA TTT CCC AAG C 3′) was designed. Thai MK-50 of CTV-*cp* gene, which is harbored in pQE30 plasmid was amplified using CTV-CP1 and CTV-CP2 primer.

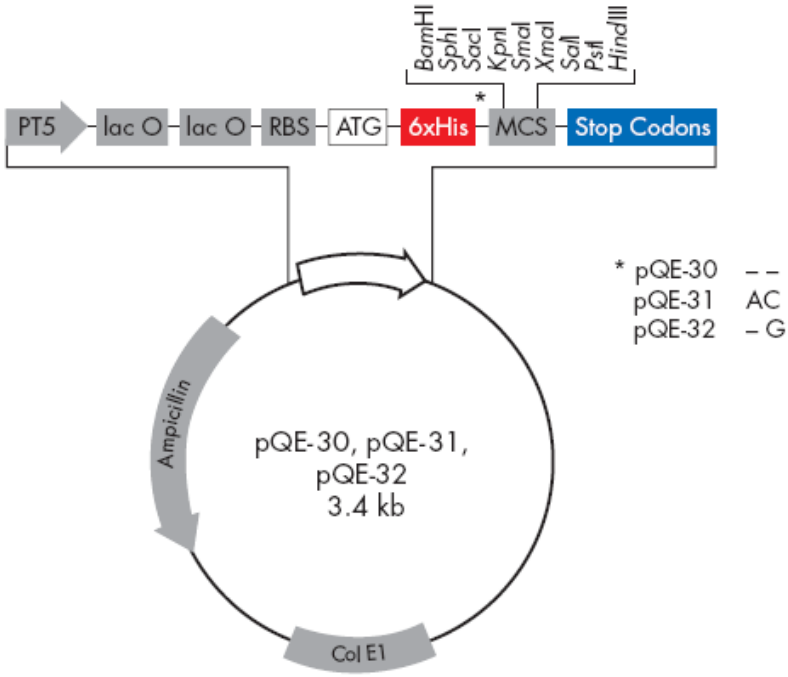

Figure 1: Schematic representation of pQE-30 vector: [**PT5**: T5 promoter, **lac O**: lac operator, **RBS**: ribosome-binding site, **ATG**: start codon, **6xHis**: 6xHis tag sequence, **MCS**: multiple cloning site with restriction sites indicated, **Stop Codons**: stop codons in all three reading frames, **Col E1**: Col E1 origin of replication, **Ampicillin**: ampicillin resistance gene, **lacIq**, lacIq repressor gene] (QIAGEN 2001).

The PCR reaction was performed in a final volume of 50 µl. The mixture contained 10 µl of 5X PCR buffer (150 mM Tris-HCl (pH 8.4), 10 mM MgCl2, 25mM β-mercaptoethanol, 0.05% gelatin and 0.5% Lubrol PX), 2µl of 2.5 mM of dNTPs, 1 µl of 20 µM of each primer (CTV-CP1 and CTV-CP2), 1 µl Phusion® DNA polymerase (Finzyme), 1 µl (50 ng) of extracted plasmid DNA of CTV-cp gene as template DNA, and 34 µl of highly deionized sterile water. The PCR program was at initial denaturing condition at 94 °C for 5 min, and 35 cycles of 1 min denaturing at 94 °C, 30 sec of

annealing at 55 °C and 45 sec of extension at 72 °C, followed by a final extension at 72 °C for 7 min. The length of the PCR product was separated by electrophoresis on 1% agarose gel in 0.5X TAE buffer along with 1 kb DNA marker.

The PCR product of CTV-*cp* was recovered from TAE gel by silica spin column (Invitrogen), and ligated into pET160/GW/D-TOPO cloning vector. The cloned plasmid (pET160-CTV) was chemically transformed into TOP10 *E. coli* competent cell for plasmid maintenance. The transformant cells were recovered in 2xYT broth for an hour at 37 °C on shaker then selected on prewarmed selective plate containing 2xYT agar media with 100 µg/ml ampicillin and incubated overnight at 37 °C (Invitrogen 2004).

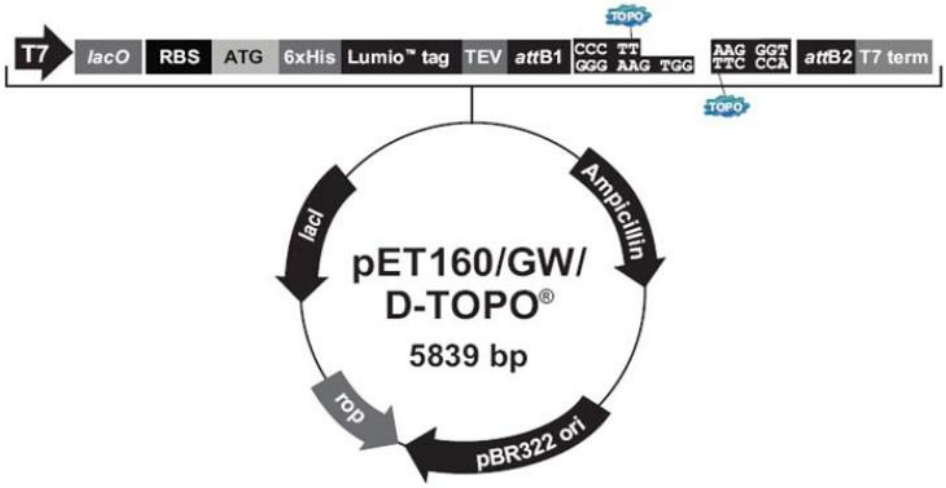

Figure 2: Schematic representation of pET160/GW/D-TOPO® (5839 bp) [T7 promoter/priming site: bases 21-40; lac operator (*lac*O): bases 40-64; Ribosome binding site (RBS): bases 94-101; Initiation ATG: bases 109-111; Polyhistidine (6XHis) region: bases 112-129; Lumio™ tag: bases 142-159; TEV recognition site: bases 169-189; attB1 site: bases 196-220; TOPO® recognition site: bases 223-236; Overhang sequence (c): bases 237-240; TOPO® recognition site 2: bases 241-245; attB2 site: bases 257-281; T7 transcription termination region: bases 307-435; T7 reverse priming site: bases 346-365; *bla* promoter: bases 740-838; Ampicillin (*bla*) resistance gene: bases 839-1699; pBR322 origin: bases 1844-2517; *ROP* ORF (c): bases 2888-3079; *lac*I ORF (c): bases 4391-5482; (c)= complementary strand.] (Invitrogen 2004)

From a number of colonies grown on selective media, six colonies were selected and cultured in test tube containing 2xYT broth media with 100 µg/ml ampicillin at 37 °C for overnight on shaker; in the following day plasmid DNA were extracted by alkaline lysis, the correct orientation of insertion was analyzed by PCR using forward primer T7F:- 5′ TAA TAC GAC TCA CTA TAG GG 3′ and reverse primer CTV-CP2:- 5′ TCA ACG TGT GTT AAA TTT CCC AAG C 3′ and sent for sequencing to "Genome Institute of Biotec". The plasmid with correct orientation was subsequently introduced into BL21 Star™ (DE3) *E. coli* expression competent cell (Invitrogen 2004), and selected on the same media as mentioned above.

Optimization of recombinant protein expression

After transforming the recombinant CTV-*cp* gene to BL21™ star (DE3) *E. coli* expression competent cell; one of the colony was selected randomly from overnight incubated plate to study the expression of CTV-*cp* gene in the expression cell, by inducing with 1 mM IPTG. Cells were collected before and after inducing with 1 mM IPTG (Invitrogen 2004).

The difference in amount of expressed protein through different period of time for recombinant CTV *cp* gene into pQE30 vector transformed to DH5α *E. coli* competent cell; pET160-CTV vector transformed to TOP10 *E. coli* cell; and pET160-CTV vector transformed to BL21™ star (DE3) of *E. coli* expression competent cell were also compared using discontinues SDS-PAGE.

Large scale recombinant protein production and purification

Large scale recombinant protein production was conducted using four one liter flask containing 250 ml 2xYT broth media consists of 100 mg/liter ampicillin with similar manner as small scale. The protein was induced with 1mM IPTG and cultured for optimum period. The induced cell collection and protein purification procedure was accomplished using QIAGEN (2003) Ni-NTA resin denatured protein purification procedure. The induced cells were collected by centrifugation at 10000 rpm, min, °C, lysed by lysozyme (concentration mg/ml) and frozen at -45^{o}C for additional 1hr, and sonicated to shear DNA and made the solution runny. The concentration of purified recombinant protein was determined by SDS-PAGE using bovine serum albumin (BSA) as a standard solution.

Results

CTV *cp* gene from Thailand MK-50 isolate was successfully amplified with CTV-CP1 and CTV-CP2 primers. The amplified gene was run on 1% agarose gel along with 1kb DNA marker and the gel photograph showed that the amplified PCR product is approximately 666 bp (Fig. 3). The amplified gene was cloned into pET160/GW/D-TOPO cloning vector and transformed into TOP10 *E. coli* for maintenance of the plasmid. The PCR result from six selected transformed colonies revealed that two of six colonies don't harbor correct orientation of insertion (Fig. 3).

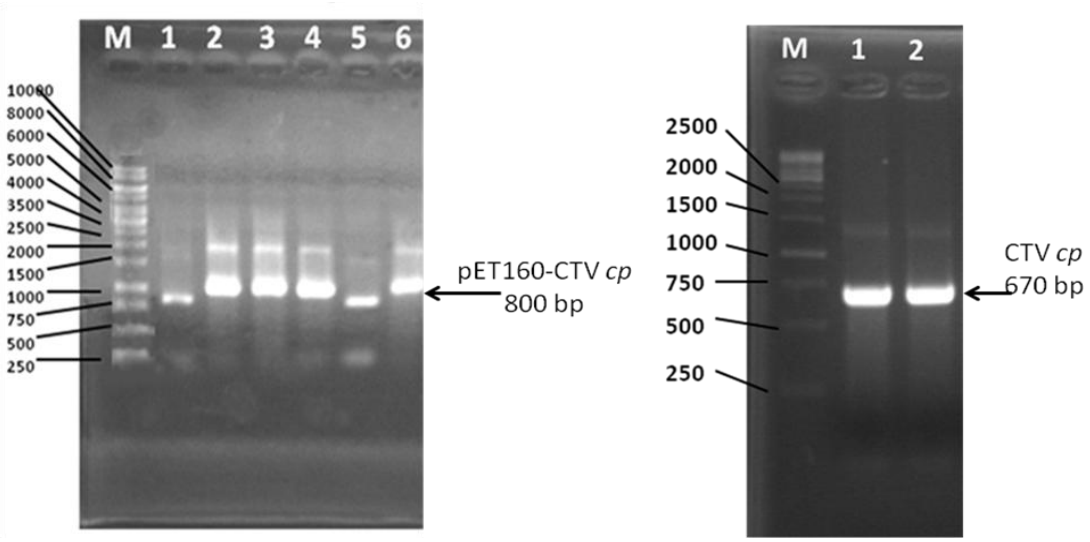

Figure 3: PCR analysis of six transformed colonies; M= 1 kb (left) 2.5 kb (right) DNA ladder, 1-6 (left)= transformed colonies CTV-*cp* into pET160/GW/D-TOPO vector amplified by T7F& CTV-CP2 primers; 1-2 (right)= CTV-*cp* gene amplified by CTV-CP1& CTV-CP2 primers

The expression of CTV-*cp* gene either in pQE30 vector transformed to DH5α *E. coli* or as pET160-CTV plasmid and in TOP10 *E. coli* revealed that the expression level was not controllable, since the expression of other bacterial protein and recombinant CTV-*cp* gene was not distinguished both before and after addition of 1 mM IPTG (Fig. 4). Therefore, for further study on expression of recombinant CTV-*cp* gene, two colonies having appropriate direction of insertion were selected and transformed into BL21 Star™ (DE3) *E. coli* expression cell, to study CTV recombinant coat protein the expression capacity.

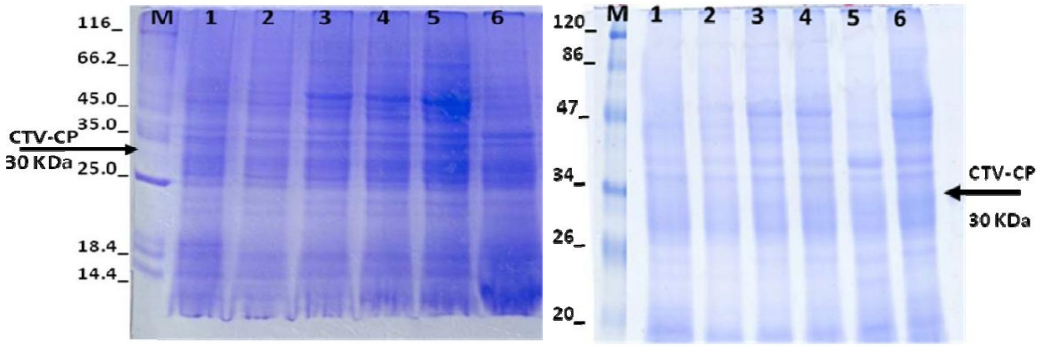

Figure 4: Expression of CTV coat protein in pQE30 vector transformed to DH5α *E. coli* (left); and in pET160-CTV plasmid transformed into TOP10 *E. coli* (right) [M= Protein Marker; 1= overnight non-induced; 2= 2 hrs non-induced; 3= 2 hrs induced; 4= 4 hrs induced; 5= 6 hrs induced and 6= overnight induced]

One of the transformed colonies plasmid was extracted and sent for sequencing and the sequence analysis result also showed that there is no deletion or insertion of bases

comparing to the sequence of MK-50 isolate (Paradornuwat *et al*. 2004). The amino acid translation of pET160-CTV-CP is presented on Figure 6. Two transformed colonies from BL21 Star™ (DE3) *E. coli* were also selected to study the expression capacity of CTV-*cp* gene. The result indicated that growing the cell for four hours or more after addition of 1 mM IPTG gave optimum amount of recombinant protein (Fig. 5). Since the difference between after four hours, six hours and overnight expression is negligible, four hours incubation after addition of 1 mM IPTG was used for large-scale production.

Since the recombinant coat protein contains 6xHistidine amino acids tag (Fig. 6), it is suitable to purify the large-scale produced protein using Ni-NTA resin column to attain a highly purified CTV recombinant coat protein used as an antigen. The molecular weight of recombinant protein was approximately 30KDa including the 44 amino acids sequences of pET160/GW/D-TOPO vector (Fig. 6), and when these amino acids were subtracted from the recombinant coat protein, the molecular weight will be 25KDa which is similar to previous research work (Nurhadi *et al*. 2001).

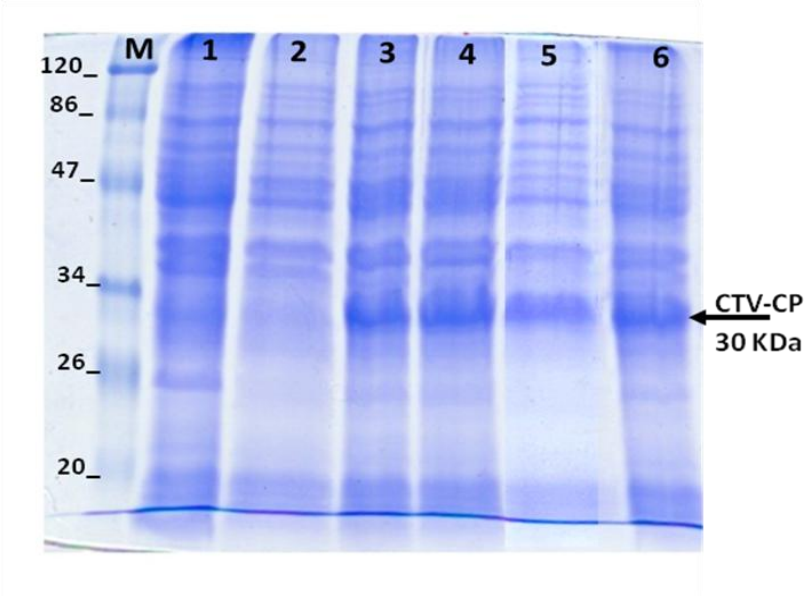

Figure 5: Expression of CTV coat protein cloned in pET160-CTV plasmid transformed to BL21 Star™ (DE3) E. coli expression cell (M= Protein Marker; 1= overnight non-induced; 2= 2 hrs non-induced; 3= 2 hrs induced; 4= 4 hrs induced; 5= 6 hrs induced and 6= overnight induced)

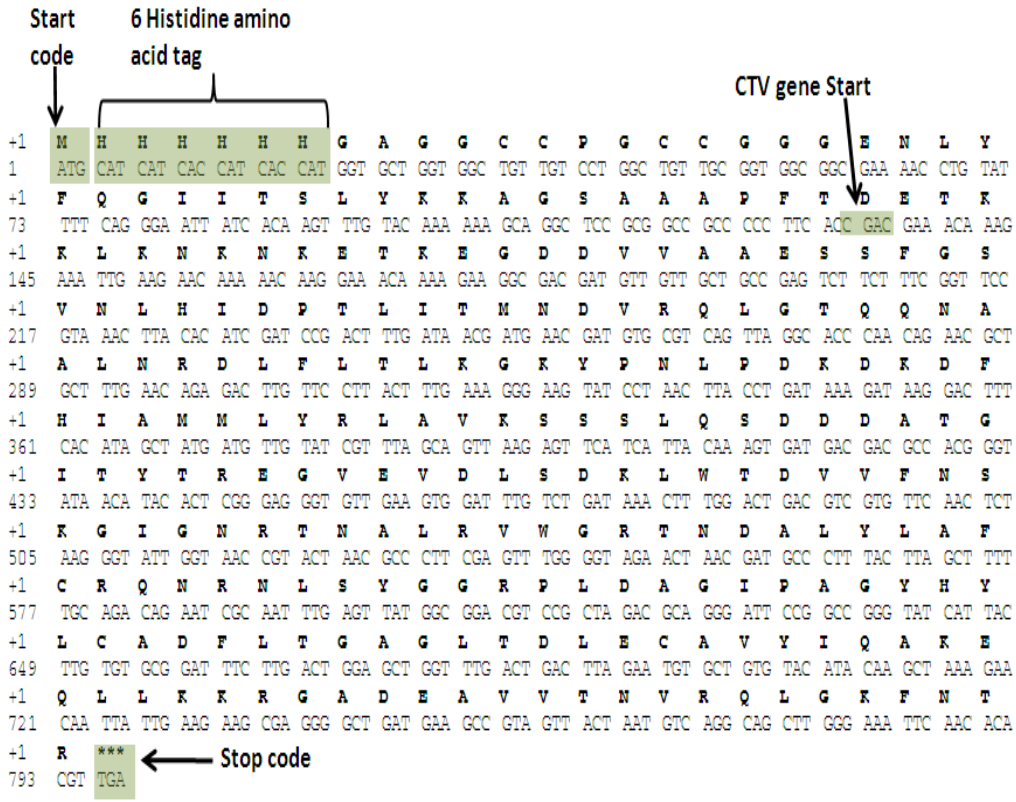

Figure 6: The amino acid translation of pET160-CTV-CP containing 798 base pairs including 666 base pairs from CTV-*cp* gene.

Since the expression of the recombinant protein from CTV-*cp* gene in pET160-CTV plasmid was expressed in BL21 Star™ (DE3) *E. coli* expression cell gave optimum level expression four hours after induction with 1mM IPTG (Fig. 5), large scale recombinant protein was produced in four one liter flasks which contain 250 ml of media.

Due to the presence of N-terminal 6XHis tag amino acid on the recombinant coat protein contains, the produced recombinant protein was purified using Ni-NTA agarose resin under denaturing condition (QAIGEN). During the purification process, the presence and amount of recombinant CTV-CP in each purification steps was confirmed by SDS-PAGE by comparing the thickness of band on gel with total amount of solution (Fig. 7).

After cell collection, lysis and sonication, the amount of recombinant protein in the crude, supernatant and pellet solution was assessed by SDS-PAGE. Even though the SDS-PAGE result showed thicker band of recombinant CTV-CP on the pellet lane (Fig. 7a), the total amount of the pellet remain after sonication was about 1-2 ml. Therefore, the total amount of recombinant protein remained in the pellet was insignificant comparing with the total amount of recombinant protein available in 25 ml of supernatant solution.

The availability of CTV recombinant coat protein from flow through, wash, and each 1 ml of fractionates of elute D (pH 5.9) and each 0.5 ml of fractionates elute E (pH 4.5) was also assessed using SDS-PAGE. The result indicate that due to the recombinant protein which possesses N-terminal 6His-tag, was successfully bound by Ni-NTA resin, there was no recombinant CTV-CP in the flow through, wash, and each 1 ml of fractionates of elute D solution (Fig. 7b and 7c). The bound recombinant protein was eluted only using low pH (4.5) elute buffer (Lysis buffer E) (QIAGEN 2003).

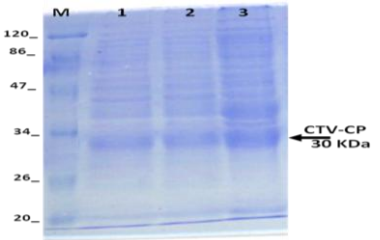

Figure 7a Large-scale production of recombinant CTV coat protein (M= Molecular marker; 1= crude protein, 2= supernatant; 3= Pellet)

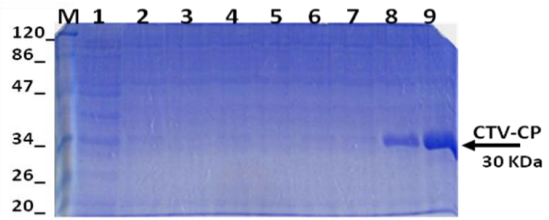

Figure 7b Large-scale production of recombinant CTV coat protein (M= Molecular marker; 1= flow through; 2= wash; 3-6= Elute D fraction 2, 5, 7 and 9 respectively; 7-9= Elute E fraction from

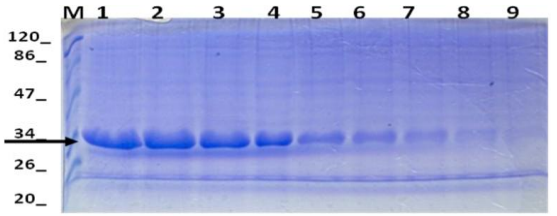

Figure 7c Large-scale production of recombinant CTV coat protein (M= Molecular marker; 1-9= Elute E from fraction 5-13 respectively)

The purified protein was dialyzed using PBS buffer and the concentration was determined using SDS-PAGE comparing with BSA as a standard and it was approximately 1.0 mg per ml (Fig. 8). A total of 4.3 mg of purified CTV-CP was recovered from one liter of bacterial culture.

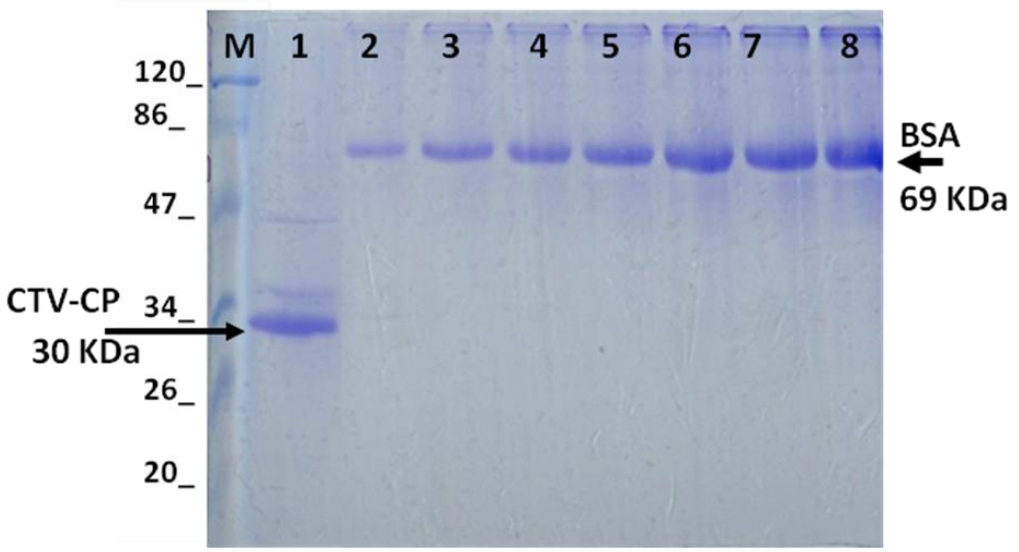

Figure 8. The protein concentration of purified CTV-CP by SDS-PAGE using BSA as standard solution **(1= CTV-CP; 2-8= (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4) mg/ml BSA respectively)**

Discussions and Conclusions

Expression of pET160-CTV-CP in BL21 Star™ (DE3) *E. coli* cell has an immense advantage over other vectors and competent cell.

(i) Cloning on this vector can be done only one times for a direct protein expression, by passing subcloning to other expression vector, and the need of ligase, post-PCR procedures, or restriction enzymes, since it contains T7lac promoter for high-level IPTG-inducible expression of the gene of interest in *E. coli*; directional TOPO cloning site for rapid and efficient directional cloning of blunt-end PCR products.
(ii) 6xHis tag for purification of recombinant fusion proteins.
(iii) Ampicillin resistance marker for selection in *E. coli*; pBR322 origin for low-copy replication and maintenance in *E. coli.*
(iv) Moreover BL21 Star™ (DE3) *E. coli* consists T7 RNA polymerase on chromosome and also regulated by *lacUV5* promoter from plasmid pET160/GW/D-TOPO. LacI regulated the expression of T7 RNA polymerase recombinant protein and expression vector and turn on simultaneously by IPTG.

Therefore, the cloning quick and simple; and the expression system is tightly regulated without leakage of basal expression (Invitrogen 2004).

The optimal time for maximum recombinant protein expression for CTV-CP pET160/GW/D-TOPO vector and BL21 Star™ (DE3) *E. coli* cell is 4 hours after addition of 1.0 mM IPTG. Similarly 4 hours after addition of IPTG was used to expression of CTV-*cp* gene in (Iracheta-Cardenas *et al.* 2002). Although the optimal time is not recommended in (Sadeghan *et al.* 2013) study, the there is no significant difference in the amount of protein expressed 3 and 4 hours after addition of IPTG.

Since the recombinant coat protein of CTV is tagged with six histidine amino acids, it was purified using Ni-NTA resin. This purification protocol was very simple but highly sensitive to pH value of the buffers. Therefore the pH of buffers used for the purification should be adjusted 30 minutes prior to use (QIAGEN 2003).

The yield of the purified recombinant protein was initially tried to estimate by Bradford's reagent but due to the protein was purified in denaturing form, it hardly dissolve in the solution and this made a difficulty to estimate the concentration of purified protein. Due to this troublesome, it was analyzed by SDS-PAGE using BSA as a standard solution (Fig. 8). The concentration of purified recombinant protein is approximately 1mg/ml and from one liter culture, a total of 4.3mg recombinant protein can be purified, which means the total amount of recombinant protein purified from a litter of culture is enough to immunize rabbit (one primary injection and six booster injection in each 500 µg of antigen was used).

After examination of Thailand CTV particles by electron microscopy and sequencing of eight isolates of CTV-*cp* gene (Paradornuwat *et al.*, 2004). Thailand MK-50 isolate is the first to be used for large scale protein expression for production of polyclonal antibodies.

Acknowledgments

The authors would like to thank, Ethiopian Ministry of Agriculture and Rural Development, Rural Capacity Building Project for their financial support to cover costs of the research expense.

References

Albiach-Martí, M. R., M. Mawassi, S. Gowda, T. Satyanarayana, M. E. Hilf, S. Shanker, E. C. Almira, M. C. Vives, C. López, J. Guerri, R. Flores, P. Moreno, G. S.M. and D. W.O. 2000. Sequences of Citrus tristeza virus separated in time and space are essentially identical. Journal of Virology 74: 6856-6865.

Bar-Joseph, M., S. M. Garnsey, D. Gonsalves, M. Moscovitz, D. E. Purcifull, M. F. Clark and G. Loebenstein. 1979. The Use of Enzyme-Linked Immunosorbent Assay for Detection of Citrus Tristeza Virus. Phytopathology 69: 190-194.

Garnsey, S. M., T. A. Permar, M. Cambra and C. T. Henderson 1993. Direct tissue blot immunoassay (DTBIA) for detection of citrus tristeza virus (CTV). Proc. 12th Conf. IOCV., Riverside, California, IOCV.

Garnsey, S. M., T. A. Permar, M. Cambra and C. T. Henderson 1993. Direct tissue blot immunoassay (DTBIA) for detection of citrus tristeza virus (CTV). Proceedings of 12th

International Conference of the Organisation of Citrus Virologists, Riverside, California, IOCV.

Harper, S. J., T. E. Dawson and M. N. Pearson. 2009. Complete genome sequences of two distinct and diverse Citrus tristeza virus isolates from New Zealand. Archives of Virolology 154: 1505-1510.

Hilf, M. E., V. A. Mavrodieva and S. M. Garnsey. 2005. Genetic marker analysis of a global collection of isolates of Citrus tristeza virus: Characterization & distribution of CTV genotypes & association with symptoms. Phytopathology 95: 909-917.

Invitrogen. 2004. Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology.

Iracheta-Cardenas, M., I. Almeyda-Leon and K. Manjunath. 2002. Molecular cloning and expression of the p25 gene of two Mexican isolates of Citrus tristeza virus. Rev Mex Fitopatol 20: 174–181.

Karasev, A. V., V. P. Boyko, S. Gowda, O. V. Nikolaeva, M. E. Hilf, E. V. Koonin, C. L. Niblett, K. Cline, D. J. Gumph, R. F. Lee, S. M. Garnsey, D. J. Lewandowski and W. O. Dawson. 1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208:: 511-520.

Mawassi, M., E. Mietkiewska, R. Gofman, C. Yang and M. Bar-Joseph. 1996. Unusual sequence relationships between two isolates of citrus tristeza virus. J. Gen. Virol. 77: 2359-2364.

Moreno, P., S. Ambrós, M. R. Albiach-Martí, J. Guerri and L. Peña. 2008. Citrus tristeza virus: a pathogen that changed the course of the citrus industry. Molecular Plant Patholology 9: 251- 268.

Nurhadi, K. Sijam and I. Sulaiman 2001. A simple purification technique for producing monospecific antigen of citrus tristeza virus. 24th Malaysian Microbiology Society Symposium. Malaysian Society for Microbiology and University of Malaya, Pahang, Malaysia.

Nurhadi, K. Sijam and I. Sulaiman. 2003. Production of polyclonal antibody to the coat protein of citrus tristeza virus in chicken eggs. Indonesian Jornal of Agricultural Science 4(1): 18-26.

Pappu, H. R., S. S. Pappu, R. F. Lee, M. Cambra, P. Moreno, S. M. Garnsey and C. L. Niblett 1994. The molecular basis for the antigenic diversity of CTV Implications for virus detection. Proc. Flo. State Hort. Soc.

Pappu, H. R., S. S. Pappu, C. L. Niblett, R. F. Lee and E. L. Civerolo. 1992. Comparative sequence analysis of the coat protein of biologically distinct citrus tristeza closterovirus isolates. Virus Genes 7: 255-264.

Paradornuwat, A. (2004). Characterization of Citrus Tristeza Closterovirus Isolates in Thailand. Plant Pathology. Bangkok, Depatrment of Plant Pathology, Kasetsart University**:** 145.

Paradornuwat, A., V. Korpraditkoon, V. Kamjaipai, S. Attatum and N. Taweechai. 1984. Citrus Diseases in Thailand. Bangkok, Funny Publishing.

Paradornuwat, A., W. Kositratana, T. Sutabutra, N. Thaveechai and S. Chowpongpang. 2004. Cloning and Sequencing of Citrus Tristeza Closterovirus Coat Protein Gene. Thai Journal of Agricultural Science 37: 99-108.

QIAGEN (2001). The QIAexpressionist™ A handbook for high-level expression and purification of 6xHis-tagged proteins.

QIAGEN (2003). The QIAexpressionist™: A handbook for high-level expression and purification of 6xHis-tagged proteins**:** 128.

Roistacher, C. N. 2004. Diagnosis and Management of Virus and Virus like Diseases of Citrus. Diseases of Fruits and Vegetables,. S. A. M. H. Naqvi. The Netherlands, Kluwer Academic. Volume I . 109-189.

Roy, A., G. Ananthakrishnan, J. S. Hartung and R. H. Brlansky. 2010. Development and application of a multiplex reverse-transcription polymerase chain reaction assay for screening a global collection of Citrus tristeza virus isolates. Phytopathology 100: 1077-1088.

Sadeghan, A. A., M. Shams-bakhsh and B. Yakhchali. 2013. Expression of Citrus tristeza virus coat protein gene in *Escherichia coli*. Journal of Crop Protection 2 (4): 387-393.

Sekiya, M. E., S. D. Lawrence, M. McCaffery and K. Cline. 1991. Molecular cloning and nucleotide sequencing of the coat protein gene of citrus tristeza virus. J. Gen. Virol. 72: 1013-1020.