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Expression of *Cucumber mosaic cucumovirus* coat protein in *Escherichia coli* and production of specific polyclonal antiserum

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ABSTRACT

Cucumber mosaic cucumovirus (CMV) was isolated from naturally infected cucumber plants serologically depending on indirect enzyme-linked immunosorbant assay (I-ELISA) and biologically by mechanical inoculated on *Chenopodium amaranticolor* as a local lesions host and maintained on *Nicotianatabacum* cv. (White Burley). Infected tobacco plant tissue used to perform immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) to isolate the viral coat protein gene (*cp*). The agarose gel analysis of the PCR product indicated a single band with 657 bplength, which is the expected size for the CMV *cp*. The isolated CMV *cp* gene has been ligated into the PinPoint™ Xa-1 T-Vector depending on the T-A cloning principle. The new plasmids were transformed into competent *Escherichia coli* cells, further the gene integration success and orientation were screened by isolation of plasmids from transformed bacteria and treatment with *Bgl*II and *Bam*HI restriction enzymes. It was found that total plasmid length was 3331 bp without insert, while fragment separated after restriction digestion was approximately 660 bp. The PCR product of the minipreparation indicated a single band with 657bp which is the expected size for the CMV *cp* gene. After induction of CMV coat protein (CP) expression using Isopropyl β-D-1-thiogalactopyranoside (IPTG), transformed bacteria lysate analyzed using SDS-PAGE revealed a band with a molecular weight of 25KDa (the expected of CMV CP molecular weight). Western blotting analysis was carried out and confirmed that all the bands expected for viral CP react positively with CMV antiserum. The induced viral CP was purified from *E. coli* transformed cells using regenerated SoftLink™ Soft Release Avidin Resin yielding about 11 mg per 1 liter culture. Polyclonal antiserum produced in a New Zealand white rabbit by injecting of the produced fusion protein and Immunoglobulins G (IgGs) were purified from the collected antiserum. IgGs titration was performed using I-ELISA, the highest titers were 1:512 & 1:256 for antisera produced using fusion protein and purified virus, respectively. It was clearly observed that purified antiserum of *E. coli* viral fusion protein was more reactive and specific as it did not have any cross reactions with healthy tobacco plant sap.

KEYWORDS

Cucumber mosaic cucumovirus;
Isolation;
Coat protein gene;
Cloning;
Protein expression;
Coat protein purification;
Antiserum production;
Immunoglobulins G purification;
Evaluation.

INTRODUCTION

Cucumber Mosaic virus (CMV, genus: *Cucumovirus*, family: *Bromoviridae*) is geographically wide spread and has been reported in Australia, North America, New Zealand, Europe and Africa[32,34]. It has four functional pieces of single stranded RNAs, packaged in icosahedral protein particles with molecular weight of 25 KDa, about 30 nm in diameter[28]. The virus is transmitted by numerous species of aphid in a non-persistent manner[5,13]. The virus has an extremely wide host range and it induces severe symptoms[8] listed 775 plant species representing 365 genera and 85 families that are susceptible to CMV infection. In Egypt CMV infects cucurbit and solanaceous crops, banana and sugar beet causing remarkable yield loss[9].

Serological methods especially the enzyme linked immunosorbant assay (ELISA) are widely used in the detection of viral infections [30]. They are relatively sensitive, inexpensive, simple and suitable for the testing of many samples simultaneously[6]. Additionally, more specific antibodies are a prerequisite for the application of the extremely sensitive technique immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR)[20,35].

Generally, molecular methods such as RT-PCR are not suitable as routine tests for indexing large numbers of samples due to costs and the relative complexity of execution. As a consequence, serology has traditionally been the most used method of plant virus diagnosis in a large number of samples, using ELISA as the method of choice[11,22,36].

Recently, advances in recombinant DNA technology, coupled with its ease to manipulate and its rapid growing rate in a less expensive media had established *E. coli* as a leading host organism to produce high protein quantities of scientific interest[2]. Many of the expression systems have advantages and limitations but *E. coli* as a prokaryotic system was used widely because of its high expression levels of many heterologous proteins, low cost, efficient generation time, and fast high density cultivation[34].

Virus purification is usually a labor-intensive procedure with varying, occasionally unsatisfactory results concerning purity and concentration of the final preparation[22]. The production of high quality virus-speciûc antiserum suitable for large scale of virus detection and based on virus puriûcation procedures faces substantial drawbacks, *i.e.*, including complex virus infections, low yields of virus particles, contamination of antigens with plant proteins, presence of inhibitory compounds[18,31]. On the other hand, it was found that the production of virus-specific antisera using recombinant proteins from cloned virus genes expressed in *E. coli* "which can overcome such difficulties" has been applied to produce a number of polyclonal antisera against coat protein (CP) of several plant viruses, *i.e.*,*Bean yellow mosaic potyvirus*[15], *Plum pox potyvirus*[26], *Soybean mosaic potyvirus*[23], *Cucumber mosaic cucumovirus*[18,34].

The aim of this study is to express CMV coat protein in *E. coli*, which was then used for the production of specific polyclonal antibodies. As antibodies with higher specificity are a crucial reagents in carrying out serological tests for screening against viruses in plant material such as seeds and other propagation material and also for field plants testing.

MATERIAL AND METHODS

Virus isolation

Fifty samples of naturally infected cucumber plants (Collected from the open field of Faculty of Agriculture, Ain Shams University, Cairo, Egypt) showing mosaic, mottling and malformation were used for virus isolation. According to[19] I-ELISA was performed on samples using polyclonal antibodies specific for CMV(Agdia Inc., USA). Samples giving CMV positive results were mechanically inoculated on *Chenopodium amaranticolor*. Local lesions produced were used as a source of single lesion isolation, and finally the virus was maintained in *Nicotiana tabacum* cv. White Burley under greenhouse conditions (28ºC ±2).

Isolation CMV*cp* gene using IC-RT-PCR

Immunocapturing,the isolation of CMV RNA and cDNA synthesis using IC-RT-PCR was performed using infected tobacco leaf collected 15 days post CMV inoculation according to[27] as follows: the wells of an ELISA plate were coated by adding 200 µl of IgGs (diluted to 10-1 using coating carbonate buffer (pH 9.6)) and incubated 4 hrs at 37°c, after that, plate was washed

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3 times with PBST, 5 min each. Plant tissues were ground in sample buffer (1 gm : 5 ml), then centrifuged at 3000 rpm/10 min at 4°c, 200 µl of the supernatant were added to each pre-coated wells and incubated overnight at 4°c, then plate was washed 3 times, 5 min each with PBST.

Twenty five of pre-heated transfer buffer (10 mMTris-HCl (pH 8.0) containing 1% Triton X-100) were added to each well and kept at 65°c for 5 min. Aliquots of 5 µl from the resulting released RNA solutions were immediately transferred to microcentrifuge tube. To each tube the following components were added and incubated at 42°c/2 h for cDNA synthesis: 1 µg of reverse coat protein gene primer, 4 µl of 5X first strand buffer (250 mMTris-HCl (pH 8.3), 375 mMKCl, 15 mM MgCl2), 3 µl of 0.1M dith-iothreitol (DTT), 5 µl of 0.3M 2-ME, 2.5 µl of 10 mM of each deoxynucleotide triphosphate (dNTPs), 1 µl RNasin (40 unit) and 1 µl of Molny Murine Leukemia Virus (M-MLV) (200 unit). Final product was used immediately for PCR reaction.

The following primers (Invitrogen Corp., USA) designed depending on CMV *cp* gene sequences collected from GenBank web site (http://www.ncbi.nlm.nih.gov) were used for the amplification of CMV *cp* gene were:

- 5'ATGGACAAATCTGAATCAAC3' (Sense)
- 5'TCAAACTGGGAGCACCCCAG3' (Antisense).

PCR was performed as described by[12] using PerkinElmer Cetus Thermal Cycler PE 9700 (PerkinElmer Inc., USA). Five µl from resulting cDNA were transferred to tube containing 45 µl PCR reaction mixture. PCR program was 94°c initial melting for 3 min followed by 35 cycles of 94°c/1 min, 55°c/1 min, 72°c/2 min and 72°c/10 min final extension.

For PCR product analysis, 1.5% agarose gel was used and electrophoresis was carried out in Sub-Cell DNA apparatus (Bio-Rad® Lab., USA) at 80 V. The amplified gene band was visualized on an UV Transilluminator and photographed by Gel Documentation System (AlphaImager® TM1220, Documentation and Analysis system, Canada).

Cloning of CMV *cp* gene

The virus *cp* gene (PCR product) was ligated with the PinPoint™ Xa-1 T-Vector (Promega Corp., USA) depending on the T-A cloning principle[16] and according to manufacturer instructions manual. The gene was cloned with the vector in-frame with the fragment of biotin-binding protein (BBP) as mentioned by Abou Zeid *et al.*[1].

The recombinant plasmids were transferred into competent *E.coli* strain BL21cells and successfully transformed bacteria were selected depending on blue/white colonies screening. Transformation and selection were carried out as described by Hanahan[14].

Minipreparation and restriction enzyme digestion

The recombinant plasmid was isolated from *E. coli* transformed cells according the miniprep procedure described by Sambrook *et al.*[33]. The preparation was subjected to restriction analysis to confirm *cp* gene orientation prior to protein expression. As described by Sambrook *et al.*[33] with some modifications, 15 µl of The recombinant plasmid was mixed with 2 µl from both *Bgl*II and *Bam*HI restriction enzymes (Promega Corp., USA), the total volume was raised to 30 µl by deionized water. The reaction was incubated at 37°c for 2 h, and then 20 µl of the digested product was mixed with 6 µl agarose gel loading buffer and electrophorised on 1% agarose gel.

PinPoint™ Xa-1 original plasmids (without the CMV *cp* gene) and recombinant plasmids with the CMV *cp* gene in addition to PCR product of the minipreparation using viral *cp* gene specific primers wereloaded to the same gel as controls.

Induction of *cp* gene expression and detection of the expressed protein

As recommended by manufacture (Promega) instructions, culture of *E. coli* carrying the PinPoint™ Xa-1 expression fusion was started by inoculating 5 ml of LB medium containing 100 µg/ml ampicillin with a 24 h colony and culture was incubated overnight at 37 °C on shaker. Overnight culture was diluted to 1:100 by adding 50 µl of culture to 5 ml of LB medium containing 2 µM biotin, 100 µg/ml ampicillin and incubated for 1 h at 37 °C on shaker. For protein expression induction, 100 µM isopropyl β-D- thiogalacto-pyranoside (IPTG) was added to culture, incubated for 4 h at 37 °C with shaking. Bacterial cells were then collected by centrifugation and further subjected to subsequent

freezing/thawing, sonication and centrifugation treatments for protein extraction[33]. The proteins were then separated by 12 % SDS polyacrylamide gel electrophoresis (SDS-PAGE)[21], electro-blotted to nitrocellulose membranes and assayed by Western blotting immunoassay using polyclonal CMV coat protein specific antibodies[17].

Protein purification

For the purification of large protein quantities, one liter of transformed bacteria culture in LB broth containing 2 μM biotin and 100 μg/ml ampicillin was prepared. The protein expression was induced by adding 100 μM IPTG to the bacterial culture and incubated 4 h at 37 °C on shaker. The cells were disrupted by sonication according to[33] and centrifuged at 8000 rpm for 10 min at 4 °C and the fusion protein was then purified using 3 ml regenerated SoftLink™Soft Release AvidinResin according to Promega instructions. The eluted fusion protein was quantified by the Bradford assay[4] at A_{595} nm using spectrophotometer. The protein was then analyzed and separated by electrophoresis in 12 % SDS-PAGE and detected using Western blotting immunoassay. As a control a purified CMV preparation was also loaded to gel.

Antisera production and evaluation

Depending on the method described by[25] polyclonal antiserum produced in a New Zealand white rabbit by injecting of the produced fusion protein and a purified CMV preparation as a control (purified as described by[24] (1 mg of virus with Freund's incomplete adjuvant, 1 : 1) intramuscularly six times at 10 days interval. The animal was bled 15 days after the last injection and the antiserum was collected.

Immunoglobulins G (IgGs) purification was carried out as mentioned by[7]. Final dialyzed proteins were loaded on 10 ml of diethylaminoethyl (DEAE) cellulose column (Whatman Inc., USA), eluted using half strength phosphate buffer saline (PBS) and collected in microtube. Absorption of fractions was measured at 280 nm and concentration of IgGs was adjusted to 1 mg/ml (OD_{280}=1.46) using half strength PBS, then stored at 4°C. IgGs dilution end points were determined using I-ELISA against clarified infected tobacco sap and healthy tobacco sap was used as a control.

RESULTS

Isolation of CMV virus

CMV was isolated from naturally infected cucumber plants showing mosaic, mottling and malformation. Samples gave positive I-ELISA results ranging from 0.897 to 1.095 were mechanically inoculated on *Ch.Amaranticolor* leaves and gave chlorotic local lesions. Lesions were extracted and inoculated on *N.tabacum* cv. White Burley for maintenance.

Isolationof CMV *cp* geneusing IC-RT-PCR technique

Infected tobacco plant tissues were used to perform IC-RT-PCR to isolate the viral *cp* gene. The agarose gel electrophoresis of the PCR product indicated

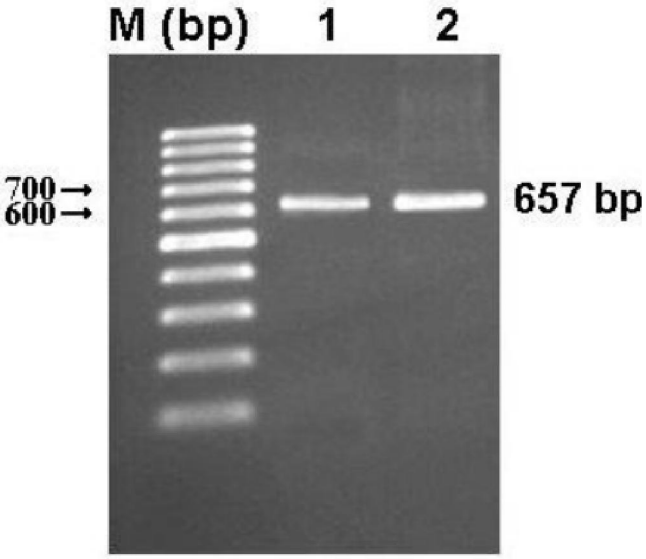

Figure 1 : IC-RT-PCR results for the isolation of CMV *cp* gene (Lane 1-2). M: DNA Ladder (Promega, USA).

a single band sized657bp, which is the expected size for the CMV *cp* gene (Figure 1).

Cloning of CMV *cp* gene

The PCR isolated CMV *cp* gene has been ligated into the PinPoint™ Xa-1 T-Vectordepending on the T-A cloning principle. The new recombinant plasmid vectorwastransformed into competent *E. coli* cells, further the gene integration success and orientation were proved by isolating the recombinant PinPoint™ Xa-1 T-Vectorfrom transformed bacteria and treating it with

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*Bgl*II and *Bam*HI restriction enzymes, which restricted to certain sites through the polylinker region of the plasmid vector. It was found that total plasmid length was 3331 bp without the insert, while the fragment separated after restriction digestion was approximately 660pbin length (gene length plus some added nucleotides after the enzymes cutting sites)compared to the PCR product using the recombinant PinPoint™ Xa-1 T-Vectoras a template, which givesa single band with 657bplength, the expected size for the CMV *cp* gene (Figure 2).

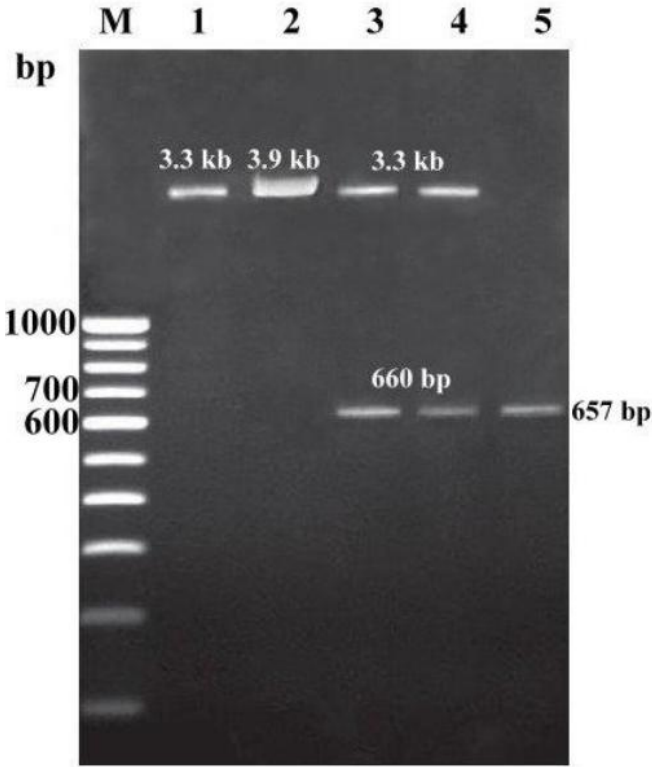

Figure 2 : Restrection analysis to chek for the right integration of the*cp* gene into PinPoint™ Xa-1 T-Vector: The emptyPinPoint™ Xa-1 plasmid vector (Lane 1), the recombinantPinPoint™ Xa-1 T-Vector (containing CMV *cp* gene)(Lane 2), the recombinantPinPoint™ Xa-1 T-Vector cut with *Bgl*II and *Bam*HI restriction enzymes (Lane 3 & 4) and PCR product for CMV cp gene using the recombinant PinPoint™ Xa-1 T-Vectoras a template (Lane 5). M: DNA Ladder (Promega, USA).

Expression, detection and purification of the CMV coatprotein transformed *E. coli* cells

Transformed bacteria lysate analyzed using SDS-PAGE revealed a band with a molecular weight of 25KDa (Figure 3A) after induction of protein expression using IPTG.

To produce large amount of the fusion protein, large scale culture from transformed *E. coli* was prepared and protein was purified depending on affinity purificationusing SoftLink™ Soft Release Avidin Resin. About 11 mg of expressed protein was purified from 1 liter of bacterial culture. The purified protein gave a band with molecular weight of 25KDa, which is expected to CMV coat protein (Figure 3A).

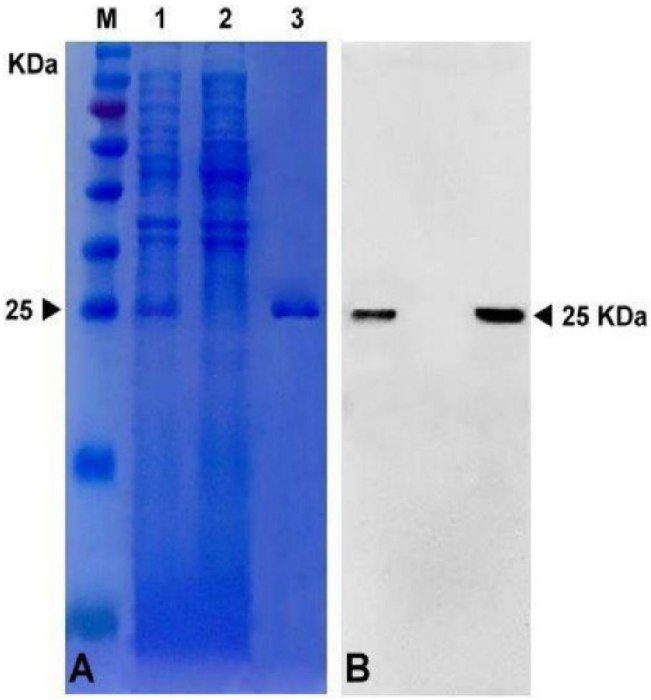

Figure 3 : (A) SDS-PAGE analysis forthe lysate of *E. coli* cells:Transformed*E. coli* lysate (Lane 1), untransformed *E. coli* lysate as a control (Lane 2),Purified fusion protein (Lane 3) M: Protein marker (Promega, USA). (B) Western blotting analysis showing positive reaction with the viral protein bands obtained from the transformed *E. coli* lysate.

Result was confirmed by transferring gel bands to nitrocellulose membrane for Western blotting analysis, and all the bands expected for viral CP (25 KDa) react strongly with CMV specific antiserum (Figure 3B).

Antisera production and evaluation

IgGs titration results were illustrated in TABLE 1, the highest titerswere 1:512 & 1:256 for antisera produced using fusion protein or purified virus preparation, respectively. It was clearly observed that purified antiserum ofviral fusion protein produced from bacterial cells was more reactive as it gave higher I-ELISA values and more specific for not giving any cross reactions with healthy tobacco plant sap.

TABLE 1 : Titration and evaluation of CMV IgGs produced from fusion protein and purified virus preparation against infected and healthy tobacco sap

Dilution		I-ELISA values at 405 nm*							
		$IgGs_1$				$IgGs_2$			
		I	R	H	R	I	R	H	R
Crude		0.914	+	0.122	-	0.820	+	0.404	+
1:1		0.825	+	0.100	-	0.801	+	0.311	-
1:2		0.810	+	0.099	-	0.766	+	0.209	-
1:4		0.805	+	0.091	-	0.677	+	0.259	-
1:8		0.787	+	0.088	-	0.660	+	0.199	-
1:16		0.731	+	0.069	-	0.611	+	0.091	-
1:32		0.711	+	0.065	-	0.589	+	0.100	-
1:64		0.677	+	0.062	-	0.579	+	0.095	-
1:128		0.649	+	0.059	-	0.490	+	0.086	-
1:256		0.550	+	0.060	-	0.441	+	0.084	-
1:512		0.504	+	0.059	-	0.064	-	0.069	-
1:1024		0.200	-	0.049	-	0.061	-	0.065	-
Purified**	1	1.353		+		1.077		+	
	2	1.042		+		0.924		+	

***Each ELISA result (R) was the average of three readings; ** Reaction of CP fusion protein (1) and crude purified virus (2) with crude antiserum; Note: Reaction of infected (I) and healthy (H) tobacco leaves sap with antisera produced by rabbit immunization with CP fusion protein ($IgGs_1$) and from purified virus preparation ($IgGs_2$).**

DISCUSSION

Cucumber mosaic cucumovirus (CMV), one of the most common virus infecting some economical plants in Egypt, the virus infects important crops like sugar beet, banana and cucurbits causing significant loss in yield quality and quantity[9,10].

Such virus is difficult to diagnose due to the periodic changes in symptoms and also for the need of high quantity of antiserum with higher specificity to perform routine serological tests for indexing large numbers of samples in field[34].

The CMV *cp* was isolated depending on IC-RT-PCR technique and then this gene has been ligated into the PinPoint™ Xa-1 T-Vector depending on the T-A cloning principle. The obtained recombinant PinPoint™ Xa-1 T-Vectorwas transformed into competent *E. coli* cells, further the *cp*gene integration was proved by restriction enzymes analysis. The PCR product of the isolatedrecombinant PinPoint™ Xa-1 T-Vector from the transformed *E. coli* cellsproduced a single band with 657bp, which is the expected size for the CMV *cp* gene. There are numerous reports of the expression of plant viruses CP in *E. coli*[15,18,23,26,34].

Transformed bacteria lysate analyzed using SDS-PAGE revealed a band of CMV CP with a molecular weight of 25KDa. Western blotting analysis was carried out and confirmed that all the bands expected for viral CP react positively with CMV antiserum. Such work agreed with that performed by Khan *et al.* and Sokhandan-Bashir *et al.*[18,34].

The induced viral CP was purified from *E. coli* transformed cells using regenerated SoftLink™ Soft Release Avidin Resin yielding About 11 mg of protein from 1 liter of bacterial culture. This result is a promising result when compared with that obtained by Khan *et al.*[18] who produced a total yield of about 8 mg CMV CP per 1 liter of cell culture.

Polyclonal antiserum produced by rabbit immunization and IgGs were purified from the collected antiserum. Depending on I-ELISA it was clearly observed that purified antiserum of *E. coli* viral fusion protein has higher dilution end point, having more reactivity for giving higher I-ELISA values, and specificity as it did not give any cross reactions with healthy tobacco plant sap which can improve the performance of the antiserum.

Barbieri*et al.* (2004) and Sokhandan-Bashir *et al.*[34] concluded, based on observations with *Watermelon mosaic potyvirus* and *Cucumber mosaic cucumovirus*, respectively that antibodies produced against expressed CPs in *E. coli* tend to be more specific, reducing the occurrence of unexpected heterologous reactions.

The use of fusion CPs as an immunogen to develop antibodies has been of great value particularly for viruses that are difficult to purify[29] and for those that are subject to degradation during the purification process[3].

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