Chapter Five
Discussion

Cucumber mosaic virus is amongst the most important of viruses to infect plants in Malaysia (Sidek, 1999). The CMV belongs to the family *Bromoviridae* and the genus *Cucumovirus*. The virus is a tripartite plus-sense single-stranded RNA virus which is easily transmissible via sap inoculation and vectors. Its wide host range is well documented and ranges from crops to ornamental plants. Its symptom varies from mild to severe, among the various strains of this virus. The virus disease is a major problem once infected as it cannot be readily eradicated.

The most important strategy remains early and accurate detection of the virus which will then allow for management strategies to be put in place. This requires not only sensitive, specific and rapid assays, but economic costs also come into play when large numbers of plants have to be tested. The standard assay most widely used is the ELISA test which is amenable to large scale and also automated applications (Lopez *et al*, 2003). One component of this assay is the production of specific yet economically affordable antibodies. In this study the use of recombinant antibodies in either bacteria or plant systems is proposed as a viable option for providing such antibodies. This strategy has been successfully used for production of antibodies used in the detection of Mollicute (Le Gall *et al*, 1998), *Citrus tristeza virus* (Terrada *et al*, 2000), *Hantaan virus* (Takakura *et al*, 2003) and Grapevine leafroll-associated virus 3 (Cogotzi *et al*, 2009).

The introduction of scFv to the development of an improved detection kit which can be produced economically would be highly favourable. In other words, infected plants can be diagnosed in an easier, more rapid and economical approach. In

conjunction with a primary disease management strategy, virus infection can be minimized.

The objectives of this research are to assess the utility of scFv derived from bacteria and tobacco plant, respectively as a source of specific antibody and to subsequently establish an immunoassay using both the scFv and the antigen produced in our laboratory. To do so, the extraction and purification processes were carried out for both systems to potentially obtain pure scFv for use in an immunoassay for the detection of CMV.

In this study, the antigen for the subsequent standardizations was the whole viral particles. Using whole virus particles would mimic in-field application for detection of the CMV virus.

Pure scFv in reasonable concentrations is also a necessary prerequisites for the assessment. Purification is important to free the scFv from contaminants such as endogenous protein, proteases, lipids, endotoxins, nucleic acids to improve the assay's sensitivity (Crowther, 2001) and to allow for standardizations in kit production.

5.1 Expression of scFv in Transgenic Tobacco Plants

Constitutive expression of the scFv in all tissues of the plant was facilitated by the cauliflower mosaic virus 35S promoter. This approach was taken to maximise the production in all cells of the plant. In this study, only the leaf tissues were used as it offered the best strategy in terms of bulk and ease of management.

The experiments carried out in this study investigated several extraction protocols which have been modified for large-scale processes, to release the scFv from the plant tissues into the extract (Foster and Taylor, 1998; Stacy and Aalen, 2000; Saravanan and Rose, 2004). All the protocols use similar extraction buffer composition. However, the protocol 3 (see Section 3.2.5.2) additionally utilized the

addition of phenol in the extraction protocol. The protein precipitation strategy was also different in all three protocols; by PEG (Protocol I), by acetone (Protocol 2) and by ammonium acetate in methanol (Protocol 3).

The three approaches showed clear differences in terms of the amount of polypeptides present in the extract and the quality of protein bands produced. In this study, F2 generation was chosen at that time due to large amount of available seed. As seen in Table 4.2, the protocol section 3.2.5.2 produced the highest total protein concentration compared to the other protocols. Also, it showed a broad range of well defined bands and low level of Rubisco in the extract (Figure 4.6). In this study, the protocol from Saravanan and Rose was adapted for large scale tobacco leaf extraction (Saravanan and Rose, 2004). This protocol is also reported to be suitable for other applications such as proteomic analysis of the plant tissues (Wang *et al*, 2003; Saravanan and Rose, 2004; Carpentier *et al*, 2005). Preparation of protein extracts by phenol has been reported to recover much higher yield than TCA, Chloroform or Ammonium Sulfate (Carpentier *et al*, 2005).

The phenol dissolves cytosolic and membrane proteins and lipids in the organic phase to be purified and concentrated during subsequent methanol precipitation. The high pH (8.5) of the buffer inhibits protein degradation which usually occurs due to endogenous proteolytic activity (Wang *et al*, 2003), neutralizes acids which are released upon vacoules rupture and lastly, inhibits ionization of polyphenols (Carpentier *et al*, 2005). The addition of SDS helps to recover membrane-bound proteins and aids the phase separation of the phenol/SDS mixture (Wang *et al*, 2003). PMSF with a half life of 30 minutes as protease inhibitor and β-mercaptoethanol as protein stabilizer were added just prior to use.

Although relatively large scale extraction was done, Table 4.2 shows consistently low levels of scFv expression in the leaves. The faint band observed from

the Western blot and dot blot further confirm the low concentrations of the plant-derived scFv. Immunoaffinity chromatography was done to further purify and concentrate the scFv. The results obtained from ELISA still suggest the low levels of the scFv in plant cells. From the data in Table 4.2 and 4.3, it was observed that there is a difference in the levels of scFv expression in F2 and F3 plants. There are many possibilities reported which cause the transgene expression instability such as transformation methods, selectable markers, transgene inheritance, components of the transgene, transgene silencing etc (Koprek *et al*, 2001; Lau and Korban, 2009). Moreover, only one line of each generation (T2 and T3) from the pool of transgenic plants were analysed for this study. Thus, this was not representative of the entire pool of the transgenic plants. Therefore in the future, more lines from several generations need to be analyzed and their expression levels can be quantified for comparative studies (James *et al*, 2004).

Future studies will also need focus on the stability of proteins in plant extracts. An alternative would be drying the leaves to obtain a tissue/buffer ratio of 1:5 which would increase protein yield in crude extracts (Benchabane *et al*, 2008). Acidic conditions in extraction buffer have also been suggested as an alternative for minimizing protein degradation (Zhang *et al*, 2006). In conclusion, an empirical assessment is needed to optimize the yield of plant-derived scFv and their structural stability.

5.2 Expression and purification of scFv in *Escherichia coli*

The most popular approach for expressing functional antibody fragments is the use of eukaryotic cells in expressing the targets at various sites. In this study, the expression vector used (pET30a-scFv clone), a specific signal sequence directs proteins to the periplasmic space. This space creates an oxidixing environment for the scFv to

be correctly processed; scFv contained intramolecular disulfide bonds and are soluble (Kipriyanov and Little, 1999). Additionally, the downstream processes such as IMAC purification is easier, with only 5 % of *E. coli* proteins present in this compartment.

Several factors which could influence the efficiency of induction of expression level of the scFv fragments were studied, such as the temperature, duration and bacteria concentration. At 30 ℃, bacteria growth at 0.6 OD_{600}nm followed by induction by IPTG for 4 hours, was sufficient for the optimal expression of scFv. It is reported that the bacterial expression systems secreting scFv fragments into periplasmic space offer typical yields of 0.1-2 mg/l in shake flask cultures (Plückthun *et al*., 1996). In this study, the yield obtained was 1±0.5 mg/l, thus within the range expected.

Sample preparation is crucial in obtaining high-quality of proteins for downstream processes. Many sample preparation methods have been reported (Wingfield, 2002). These methods normally are used to complement the purification steps, if needed. Additionally, there is a critical need for a rapid and effective protocol to obtain a pure scFv, for subsequent functional analysis. In this study, four steps under evaluation were the bacterial lysis step, buffer selection, type of affinity-column chromatography and elution steps.

In this study, the addition of lysozyme to the cell lysate for 30 minutes (Protocol 3) proved to be effective in releasing the scFv from the bacteria cells. Addition of DNase and RNase proved to be dispensible if the duration of enzymatic lysis was 30 minutes. Any delay would release the nucleic acids to compete with the scFv in binding onto the column (data not shown) where a viscous extract was observed. The freeze-thaw cycle (Table 4.5) did not appear to effectively release the scFv from the periplasmic space. Moreover, this physical lysis could be difficult to perform in larger scale such as in 250 ml centrifuge tubes as carried out in the laboratory. For economical sample preparation to be done with minimum requirement of equipment in

this study, selective lysis using the lysozyme proved to be feasible while the 30 minutes time frame appeared to be sufficient to release the periplasmic-space-scFv (Wingfield, 2002).

Another aspect to consider would be the type of buffer for scFv which is suitable for extraction, purification and immunoassay. In this study, three buffers were tested i.e, PBS, Tris-HCL and binding buffer. These buffers differed in terms of chemical compositions (see Appendix B and E). In Table 4.5, a concentration of 0.367 mg/ml of total protein samples was obtained using protocol 3 which used the binding buffer and was higher in yield compared to other buffers. Therefore for the extraction and purification steps, the binding buffer was used. Due to the fact that the scFv developed for this study was tagged with Histidine, affinity chromatography could be carried out to isolate relatively pure scFv. The study also demonstrated that the presence of 0.5 M of sodium chloride aided in the binding of scFv to the column.

Additionally for the purification, a two step purification using Ni-chelate chromatography was also investigated. This method has been reported to give sufficiently pure products for use in other analytical procedures. One step purification using Ni-chelate chromatography is normally used (Brereton *et al*, 2007; Tsai *et al*, 2008; Xiong *et al*, 2009) however for this study, the same step was repeated after dialysis to obtain a much purer scFv.

A comparison was carried out between the self-constructed Ni-NTA agarose (Qiagen, USA) column and His Gravitrap^{TM} column (Amersham Biosciences, USA). The binding of the scFv appeared to be stronger to the His Gravitrap^{TM} column compared to the self-constructed Ni-NTA agarose column. This can be inferred from the data in Table 4.6 which showed that the concentration of the scFv recovered by His Gravitrap^{TM} column was significantly higher ($t=37.2$, $p<0.05$, $df=4$) than that of the Ni-NTA agarose column. Additionally, the self-constructed Ni-NTA agarose column

needed to be recharged each time after use which presented additional steps and time to the protocol.

Another important parameter to be considered is the elution steps. In this study, two aspects were analyzed; elution using low pH and addition of imidazole. The former confers a positive charge on the His residues so that they are incapable of binding metal ions while the latter competes with His for metal binding.

Using the His gravitrap column, the low pH washing steps (pH 6.3) could not effectively separate the scFv from the other proteins. The SDS-PAGE (Figure 4.14) showed that the washing steps only eluted a little of unspecific proteins while most of the proteins were still intact and were recovered with low amounts of the scFv during the elution steps. Therefore, elution using low pH was not used further.

It was also shown in the SDS-PAGE analysis (Figure 4.15A) that 10 mM of imidazole alone could not effectively separate the scFv from the other proteins. However, addition of 20 mM imidazole into both the binding buffer and washing buffer appeared to help eliminate unspecific binding of proteins to the resin as well as binding of nucleic acids to the scFv (Figure 4.17 and 4.18). Intact, pure scFv with molecular weight of 42 kDa was then confirmed by Western (Figure 4.19) and Dot Blot (Figure 4.20) analysis. Therefore an optimised protocol is obtained and summarised in the flow diagram in Figure 5.1.

The use of ELISA to test the functionality as well as the sensitivity of purified scFv was investigated initially with a simple series of dilutions. The positive results obtained confirmed that the scFv could be further used for assay validation using checkerboard titration (Figure 4.21).

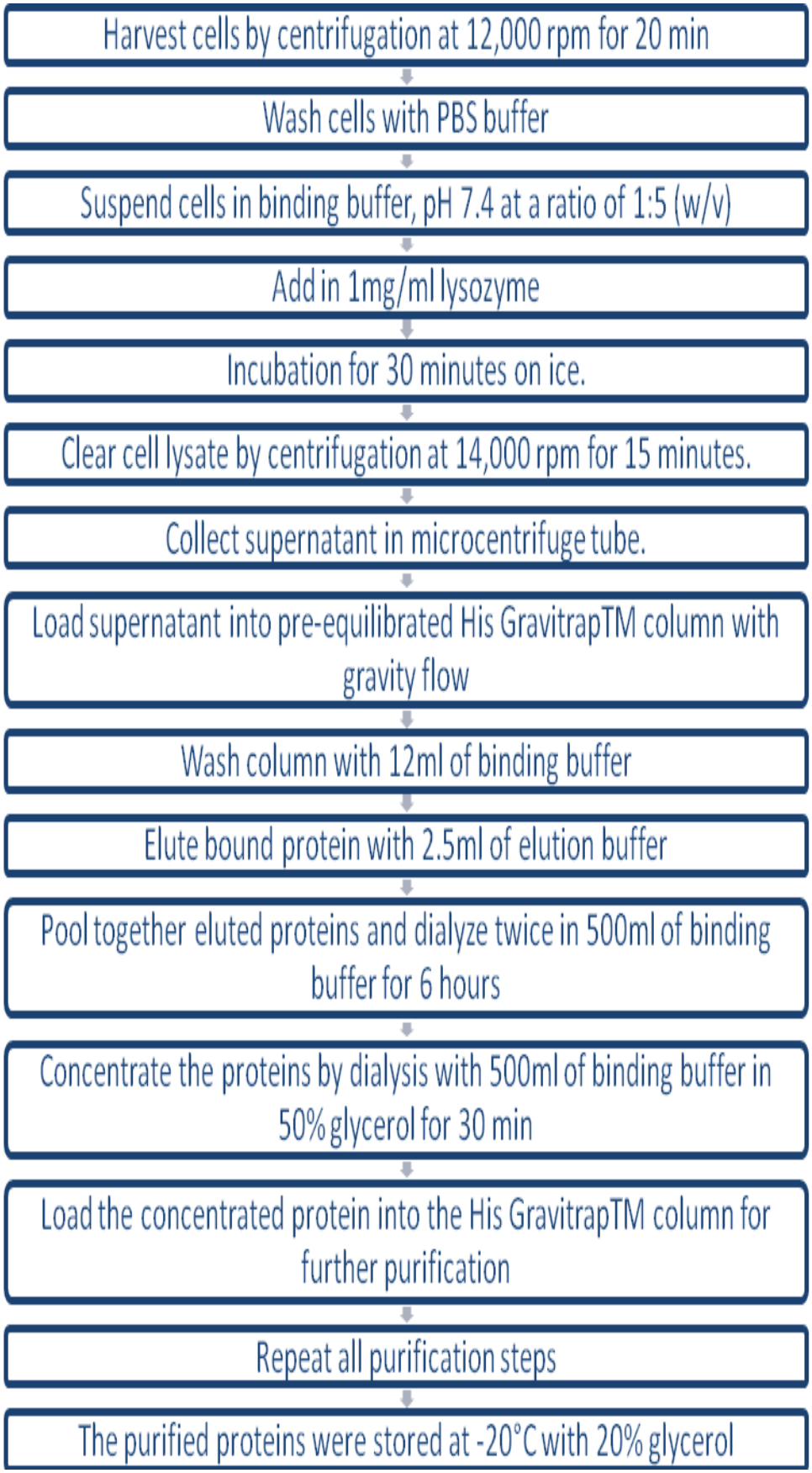

Figure 5.1 : Flow Diagram of scFv purification

5.3 ELISA Assay

Diagnosis of infectious disease by immunological tests is a useful method to test for many conditions using minute amounts of samples. The accurate and sensitive measurements of antigens by immunoassays have broad applications in agriculture. Several direct and indirect methods exist for diagnosing antigen in plants. The most commonly used immunoassay platform is the enzyme linked immunosorbent assay (ELISA). ELISA is often chosen for diagnostic purposes as it is relatively easy to set up and use.

Recombinant ELISA system based on antibody fragment has been exploited as an alternative to assays, which use monoclonal or polyclonal antibodies (Kerschbaumer et al, 1997). The antibody fragments can be engineered as coating or detecting reagents (Smith and Benfey, 2002; Takakura et al, 2003; Ushio et al, 2008). In addition to its high specifity a distinct advantage of this type antibody for production of kits is its relative stability and ease of production.

The scFv as capture antibody was prokaryoticly expressed in a soluble form and purified using an optimised process as described after which a DAS-ELISA method was applied for detection of *Cucumber mosaic virus* as the target antigen.

In addition to the purified scFv, to validate the ELISA test a purified form of the CMV antigen was also required. The antigen used in this study was whole virus particles raised in tobacco plants. From the results in Figure 4.23 and Table 4.7, the saturated binding of scFv was observed at the dilutions ranging from 1:1(1.5 mg/ml) to 1:100(15 µg/ml) while for CMV was from 1:50(0.03µg/µl) to 1:100(0.015µg/µl). The use of high concentrations of both reagents has been suggested to affect the sensitivity of the assay. However as the concentration of both reagents was reduced, decrease in the interaction binding between the scFv and antigen was significant. In other words, the use of lower concentrations may result in difficulty of detection of the antigen, due

to the low binding level of the scFv. Therefore, it is concluded that the optimal concentrations of the scFv and CMV are 7.5 µg/ml respectively where the C4 well showed optimal OD value (see Table 4.7).

The efficiency of the use of the scFv in the ELISA assay could be improved further. For example, modification to the scFv by attaching to a fusion protein such as Alkaline Phosphatase (Kerschbaumer *et al*, 1997) or Green Fluorescent Protein (Cao *et al*, 2008) could eliminate the need for the second antibody. Although the specificity of this antibody was tested against a number of viruses in a previous study (Chua, 2002) further specificity tests of the scFv against other isolates of CMV and other plant viruses has to be carried out to measure the extent of cross reactivity between other closely related and unrelated antigens (Koenig, 1978; Koenig, 1981; Zimmermann and Regenmortal, 1989)