Rabies remains a major public health problem worldwide and causes around 70,000 human deaths each year (Liu and Ertl, 2012; www.rabiescontrol.net/news/news-archive/annual-number-of-deaths). Over 95% of these human rabies cases occur in the developing nations of Asia (especially in India) and Africa (Bourhy et al., 2010). Prevalence of rabies in India is reported to be the highest in the world. Tens of thousands of human cases occur each year from the bites of rabid dogs, but inadequate reporting of rabies cases grossly underestimates the overall magnitude of the problem. In India around 20,000 human deaths are reported every year due to rabies (www.who.int/bulletin). Rabies is still a fearful disease with almost 100% mortality rate. In addition to mortality, rabies poses a major economic burden as a result of high cost of PEP in humans and loss of livestock animals.

The disease is caused by RV, a neurotropic RNA virus belonging to the genus Lyssavirus of the family Rhabdoviridae. The genus Lyssavirus can be subdivided into seven genotypes on the basis of the sequences of the complete N gene (Bourhy et al., 1993a; Gould et al., 1998), or the gene encoding the transmembrane glycoprotein (Badrane et al., 2001). RV is a negative stranded RNA having five structural proteins (3’ to 5’): nucleoprotein (N), phosphoprotein (P),
matrix protein (M), glycoprotein (G) and RNA dependent RNA polymerase (L) (Wunner et al., 1988). The G protein coding region consists of a well conserved ectodomain, variable transmembrane domain and an endodomain (Tordo et al., 1993c). The endodomain interacts with internal proteins (Mebatsion et al., 1999). The G is the only RV protein that projects through the envelope which is the major target for neutralizing antibody responses (Wiktor et al., 1973). It is the mediator of both, binding to cellular receptors and entry into host cells. The RVG also carries B- and T-cell antigenic sites and is highly immunogenic protein that protects against the RV infection (Lentz et al., 1982; Tuffereau et al., 1998; Gaudin et al., 1999).

Rabies is a fatal zoonotic disease. Animals and human beings usually acquire infection following a bite by a rabid animal (Hemachudha et al., 2002). Less often, an animal or person is infected by contact with infectious saliva or neurological tissues, through mucous membranes or breaks in the skin. The rabies virus is not transmitted through intact skin. There are also other rare routes of transmission through inhalation of aerosols; ingestion of infected animal meat and milk; and organ transplantation (Gode and Bhide, 1988; Krebs et al., 1995; Hellenbrand et al., 2005; Smith and McDonald, 2006; Dutta, 1998; www.cfsph.iastate.edu/Factsheets/pdfs/rabies.pdf). The incubation period of rabies is 1–3 months but may range from less than 7 days to more than 6 years (Smith et al., 1991; Hemachudha and Phuapradit, 1997). The early
clinical signs may include nonspecific prodromal signs such as malaise, fever or headache, as well as discomfort, pain, pruritus or sensory alterations at the site of virus entry. After several days, anxiety, confusion and progress to insomnia, abnormal behavior, hypersensitivity to light and sound, slight or partial paralysis, hypersalivation, difficulty swallowing, pharyngeal spasms upon exposure to liquids, and convulsions. Either an encephalitic form with hyperexcitability, autonomic dysfunction and hydrophobia, or a paralytic form characterized by generalized paralysis, may predominate. Death usually occurs within 2 to 10 days, survival is extremely rare (Hemachudha and Phuapradit, 1997; Hemachudha and Mitrabhakdi, 2000). Domesticated animals and individuals at risk should be vaccinated against rabies to prevent them from becoming infected. PEP should be started immediately after the exposure as there is no effective treatment available once the symptoms develop.

Though all mammals are susceptible to rabies, a comparatively small number of carnivores such as dogs, foxes, raccoons, raccoon dogs, skunks, and mongoose are recognized as the main reservoirs. In addition, bats have also been known as a primary reservoir from which rabies spread to terrestrial animals. Although eradication of rabies virus is not an easy task, vaccination of the primary terrestrial vector species is the most effective method of controlling rabies (Freuling et al., 2009). In the developed countries, human rabies has dramatically declined during the past 50 years by regular vaccination of pet animals. On the other hand, wildlife rabies has emerged as a
major threat (Rupprecht et al., 1995). Thus, controlling rabies and protecting humans from rabies requires routine vaccination of pet and wildlife animals. There is a demand for rabies vaccines not only for rabies epidemic areas, but also for the rabies-eradicated countries to maintain their rabies-free state.

5.1. Rabies vaccines

Since the Louis Pasteur's first successful rabies vaccination (Pasteur, 1885) significant progress has been made to improve the efficacy of rabies vaccines (Dietzschold et al., 2003). Although, the development of a rabies vaccine for humans has an eminent history of more than 120 years, rabies is still ranked as a seventh most important infectious disease. Rabies remains a global health threat despite it being a vaccine preventable disease. This status clearly indicates a demand for the more effective and economic rabies vaccines (McGettigan, 2010). The nervous tissue derived or cell culture derived rabies vaccines are mainly used for the pre and post exposure vaccination of humans and domestic animals. On the other hand, oral rabies vaccines have proven to be the only effective rabies control method for wild animals.

The rabies vaccine manufacturing technology has evolved from the usage of crude animal tissue homogenates (nervous tissue vaccine) to the cell culture derived highly purified vaccines. Nervous tissue derived vaccines had poor immunogenicity and safety related issues which have increased the risk of vaccine failure (Perrin et al., 1999). Thus, numerous efforts were made for the development of cost-
effective, high potency rabies vaccines. Cell culture derived inactivated rabies vaccines are much safer with excellent potency than the brain tissue vaccines (Sureau, 1992). They can be administered as prophylactic vaccines as well as post exposure treatment of the people who were bitten by rabid animals. Since, the number of rabies vaccine manufacturer’s are limited, the availability of inactivated rabies vaccine is a major concern in many of the developing countries and also the cost of the cell culture vaccines are prohibitive (Horack, 1939; Sureau, 1992). Moreover, the manufacturing process of the cell culture vaccine poses biosafety concerns due to the handling of large amounts of infectious materials (Winkler et al., 1973).

Generally, oral rabies vaccines are either of modified live attenuated rabies viruses originating from the strain SAD (Wilhelm and Schneider, 1990; WHO, 2005) or of a genetically engineered modified live vectored vaccine by inserting the RVG gene (e.g. vaccinia virus, adeno-viruses) and to use them as a live vaccines (Kieny et al., 1984; Paoletti, 1996). Live attenuated RV vaccine (SAG-2) and VRG have been developed and licensed, for oral immunization of wild animals (Paoletti, 1996; Rupprecht et al., 2004). These vaccines are effective in wild animals, however, human exposure to VRG may cause intensive skin inflammation and systemic vaccinia infection (Rupprecht et al., 2001; CDC, 2009) and SAG-2 induces low VNA responses (Hanlon et al., 2002). Other genetic vaccines (Wunner and Briggs, 2010) and gene deficient rabies vaccines are also developed and evaluated as a live attenuated vaccine candidate (Shoji et al.,
However, the live rabies vaccines bear the intrinsic risk of mutating or reverting to a more virulent phenotype. Thus, there is a need for low cost rabies vaccines with high safety and efficacy which could be produced in a GMP facility without biosecurity.

Another approach for rabies vaccine is based on the fact that G of RV itself is enough to induce protective immunity (Wiktor et al., 1973). Therefore, the sub-unit vaccines developed using RVG have been found successful in inducing neutralizing antibody response and protecting mice against i.c rabies challenge (Fries et al., 1996; Xiang et al., 1994; Badrane and Tordo, 2001; Dietzchold et al., 1978; Ertl, 2009). With the advances in biotechnology it is now possible to express the individual viral antigens in large amount using highly efficient prokaryotic or eukaryotic protein expression systems without having to handle the live RV.

The RVG has been expressed in various expression systems and the potential immunization with the expressed rRVG has been evaluated. The G expressed in E.coli and yeast resulted in an incorrectly folded version which could not protect against an i.c. challenge with the virulent rabies virus (Klepfer et al., 1993; Lathe et al., 1984). Among the other available expression systems, BEVS has become one of the most widely used, powerful and versatile eukaryotic expression systems for the production of recombinant proteins in insect cells (Lucknow and Summers, 1988; Kost and Condereay,
Advantages of BEVS over bacterial expression systems are that mammalian proteins expressed in insect cells undergo eukaryote-specific post translational modifications. Therefore, the protein folding and processing in BEVS are more native compared to other prokaryotic expression systems albeit the differences in glycosylation (Lucknow and Summers, 1988; Prehaud et al., 1989). The RVG expressed using the BEVS were antigenically conserved with similar three dimensional structure and biological features with those of the native protein (Prehaud et al., 1989; Tuchiya et al., 1992).

5.2. Characterization of BEVS expressed rRVG

In the present study, the RVG was expressed in BEVS (rRVG) and the quantitative and qualitative studies of expressed rRVG were performed by FACS, Immunofluorescence, confocal microscopy and immune-blotting techniques. The profile obtained by immune-blotting revealed the existence of a doublet rRVG (G1 and G2) showing distinct electrophoretic mobilities probably due to different glycosylation levels or truncation of protein during translation (Figure 4.4b). Similar phenomena with different electrophoretic mobility and production of heterogeneous species of G were also reported in the case of BEVS expressed rRVG of CVS and Nishigahara strains of RV (Prehaud et al., 1989; Tuchiya et al., 1992). In general the requirements for glycosylation are more stringent for membrane proteins than for secretory proteins. Glycosylation patterns of recombinant proteins are related for the immunogenicity, pharmacological activity,
pharmacokinetic profile, solubility and stability of the protein (Devasahayam, 2007). Though the glycosylation of recombinant proteins produced in insect cells may not be similar to those produced in mammalian cells, the biological activity of BEVS expressed proteins are not compromised in most of the experiments (Prehaud et al., 1989; Coleman et al., 1997). The specific binding of mAb (M5B4) with Sf-9 cells expressing rRVG in flow cytometry and confocal microscopy confirms the natively folded conformation of the BEVS expressed rRVG (Prehaud et al., 1989). Cellular locations and surface expression of rRVG in Sf-9 cells were determined by immuno-fluorescence tests. Earlier studies on RVG suggested that correct folding is a prerequisite for the surface expression of G and also for the maintenance of biological activity (Wiktor et al., 1984). The typical membrane fluorescence of the rRVG expressing Sf-9 cells indicated that the recombinant G was processed normally, transported and anchored onto the plasma membrane similar to that of native viral G protein (Prehaud et al., 1989).

5.3. Membrane extraction of rRVG

RVG is present in trimeric form. Expressing the RVG with TMD is essential to preserve its trimeric structure which is responsible for the consistent induction of virus neutralizing antibodies and protection (Wiktor et al., 1973; Desmezieres et al., 2003). However extraction of membrane bound G from the host cells without altering its conformation and antigenicity is a difficult task. Since the BEVS expressed rRVG is membrane bound, attempts were made to isolate
the membrane protein which is a tedious, time-consuming and cumbersome process. Most of the membrane protein solubilization methods result in poor protein yields (Morre and Morre, 1989). Detergents are commonly used in biological research to isolate, solubilize, and manipulate membrane proteins for subsequent biochemical and physical characterization (Garavito and Jenkins, 1983). Cell lysis using detergents is a milder and easier alternative to release the membrane bound proteins. Although many new detergents are currently available for use with membrane proteins, their behavior in solution and in the presence of protein may limit their use with specific experimental techniques. Hence the choice of detergent and experimental conditions will have an enormous impact on whether a technique can be successfully applied to a specific membrane protein extraction. Detergents break the lipid layer surrounding the cells by solubilizing the proteins and disrupting the lipid: lipid, protein: protein and protein: lipid interactions.

It is difficult to classify detergents into denaturing and non-denaturing types. However, the zwitterionic (CHAPS), and nonionic (Nonidet P-40 and Triton-X) series of detergents are commonly used for these purposes as they are milder and less denaturing. In contrast, ionic detergents are strong solubilizing agents and tend to denature the proteins, thereby destroying their activity and function (Helenius et al., 1979). In addition, the choice of detergent for cell lysis also depends on the sample type. Animal cells, bacteria and yeast cells have different requirements for optimal lysis due to the presence
or absence of a cell wall. On the other hand, effective cell lysis and protein extraction from different cells and tissue types require not only the correct choice of detergents but also buffers. Thus, suitable buffers are required for the detergents to effectively penetrate between the membrane bilayers to form mixed micelles with isolated phospholipids and membrane proteins (Tanford and Reynolds, 1976; Neugebauer, 1990). Therefore, attempts were made to prepare combinations of three detergents with six buffers (totally 18 buffered detergent solutions as shown in Table 3.2) for effective solubilization of membrane bound rRVG from Sf-9 cells. The zwitterionic CHAPS detergent is a sulfobetaine derivative of cholic acid which is non-denaturing and effective in disaggregating the membrane protein. Triton X-100 is a nonionic and non-denaturing, but appears to be inefficient in breaking protein-protein interactions (Helenius et al., 1979). Our results had also shown that the detergent CHAPS in combination with buffer-1 could yield the highest amount of soluble G compared to other detergents used in this study (Figure 4.9).

Though the use of detergents may have beneficiary effect for initial cell lysis or membrane protein extractions, subsequent applications or experiments with the extracted proteins may require removal of some or all of the detergent. Hence selection of an ideal detergent should mainly depend on the intended application of the extracted protein. The CMC is the minimum concentration of detergent at which micelles form. CMC is also an indicator of the strength of detergents to bind to proteins; i.e., low CMC values
Detergent monomers bind to water-soluble proteins at concentrations below the CMC. Binding of detergent to proteins competes with the self association of detergent molecules into micelles above the CMC. Consequently, there is effectively no increase in protein-bound detergent monomers with increasing detergent concentration beyond the CMC. The CMC is also influenced by pH, ionic strength, temperature, and impurities in the sample solution. The CMC values reported in literature are therefore appropriate for the given conditions (Neugebauer, 1990; Hjelmeland, 1986). In addition, the amount of detergent needed for optimal protein extraction also depends on the nature of detergent, aggregation number, temperature,
and nature of the membrane. The solubilization buffer should contain sufficient detergent to provide greater than 1 micelle per membrane protein molecule to ensure that individual protein molecules are isolated in separate micelles (Helenius, 1979). Thus further experiments were carried out to find the suitable concentration for the efficient solubilization of rRVG using CHAPS-buffer-1 combination. The concentration of 0.4% to 0.7% of CHAPS at RT (23-25°C) had shown superior solubilization effect when incubated for 30 min to 1 hr. The concentration optimized is almost closer to the standard CMC of the CHAPS detergent (8-10mM or 0.5-0.6%).

DMSO is a small amphiphilic molecule which is widely employed in cell biology. The role of DMSO in enhancing the solubilization of rRVG is not clearly understood. We speculate that the ability of DMSO to induce pores on cell membranes (Andrey et al., 2007) as one of the reasons for the enhanced solubilization by facilitating the cell lysis.

Detergent cell lysis is a milder and easier alternative to physical disruption of cell membranes although it is often used in conjunction with homogenization, mechanical grinding, sonication and freeze thaw. In this present study also, various sonication and freeze thaw procedures were evaluated in combination with the detergent treatment as shown in table 4.2. The sonication and freeze thaw steps in general helps to release the recombinant proteins located in the cytoplasm of cells by breaking open the cell wall. The inclusion of sonication and freeze thaw steps have not shown any enhanced effect
on solubilization of the membrane bound rRVG (Table 4.2 and 4.3). Astray et al., (2008) has also reported that the effectiveness of physical cell lysis procedures (sonication and freeze thaw) on solubilization of RVG is comparatively lesser than the chemical lysis methods using detergents.

5.4. Purification of rRVG

Expression of recombinant proteins in mammalian cell lines has become an important approach. On the other hand, it is often difficult to recover and purify the recombinant proteins from mammalian cells. Therefore the use of fusion proteins incorporating affinity tags has become more widespread for the ease of purification of the expressed recombinant proteins. But the recombinant proteins intended for animal or human vaccination should be devoid of any affinity tag to meet the regulatory requirements. For that reason, rRVG was also expressed without C-terminal His$_6$ tag in BEVS for the subsequent in-vivo use. This necessitated an alternative method for the purification of affinity tag free RVG without altering its immunogenic conformation.

IAC on the other hand, is an efficient method allows a single step purification of the antigen from the complex cell lysate due to the high avidity and specificity of the mAbs coupled with the resin. This involves the selection of suitable antibodies, optimal coupling and elution conditions for the dissociation of the target protein in a pure, stable and active form. A study was undertaken to develop an
immuno-affinity chromatographic method for the purification of rRVG using a mAb, M5B4 which binds to antigenic site III of RVG.

Due to their customized avidity and specificity, mAbs have become indispensable for both protein characterization and purification (Subramaniam et al., 2002). However affinity of the mAb to its antigen is the most important factor to be considered while using the mAb for the IAC. The low affinity mAbs have reduced binding capacity for the target antigen whereas the high affinity mAbs require very harsh elution conditions. Thus mAbs with medium to high affinity are considered as better choices for immuno-affinity purification (Pepper, 1992; Ryu et al., 2000). Affinity constant of $2.06 \times 10^{-7}$ M was obtained for the mAb at equilibrium making it suitable for IAC. This value assured a sufficient affinity for selective binding of the ligand which permitted the use of mild elution conditions.

The medium affinity mAb-M5B4 was coupled with the NHS-activated Sepharose resin as recommended by the manufacturer. A coupling pH range of 6-9 was recommended as the NHS ester groups rapidly hydrolyse at higher pH. Around 93 to 95% of the mAb were coupled at a pH of 8.3 using ice-cold buffers similar to mAb coupled with CNBr-activated sepharose as reported by Hernandez et al. (2001). mAb coupling efficiencies in the range of 75–95% are generally acceptable in IAC (Subramanium et al., 2002). The concentration of 10 mg/ml of the ligand (mAb-M5B4) as employed by Gallant et al.
(2008) is favored for its appropriate penetration, homogeneity and distribution in the Sepharose matrix during coupling.

Some antigens require more or less harsh conditions for dissociation from an immobilized mAb. An ideal elution buffer for IAC should effectively release the protein without abrogating the function of the protein. Therefore conditions for elution may have to be determined experimentally. Acid elution is the most widely used effective method of desorption of bound antigen (Subramaniam et al., 2002). Among the elution buffers tested, the 0.1M glycine with pH 2.5 could elute 80% of bound proteins. Acid elutions were neutralized by a suitable buffer of alkaline pH to avoid acid-induced protein denaturation. The results demonstrated that the microplate assay can be very useful for the initial evaluation of a large number of buffers to recover the maximum bound antigen as reported by Agraz et al. (1994) and Ibarra et al. (1999). The use of low pH elution buffer (0.1M glycine, pH 2.5) does not denature the protein when the eluates are verified in IC-ELISA and this result is in agreement with the earlier report (Santucci et al., 1990).

The chemical compound released upon reaction of the activated ester and reactive amine to form a stable amide linkage interferes with the $A_{280}$ measurement and hence measuring the $A_{280}$ of the supernatant cannot be reliably used to check the mAb leaching during the purification process (Subramaniam, 2002). This issue required the use of the antibody-mediated ELISA standardized in-house for analyzing the mAb leaching from the column. The amount of
mAb leaching in all column washes and eluates as measured by the antibody-mediated ELISA was minimal. This is in agreement with the findings reported by Chase, (1983) that NHS activated support shows reduced antibody leaching compared to CNBr-activated supports.

Although the Sepharose gel by itself can resist elevated flow rates, a diffusion mechanism probably limited the antigen accessibility to the mAb molecules in the beads, considerably lowering the functionality of the coupled ligand as reported earlier by Agraz et al. (1994). On the other hand, the observed phenomenon of recovery seems to be the result of an efficient mass transfer of rRVG into the gel, but with a limited binding of the antigen to the coupled gel i.e., not more than 40 µg/ml. Steric occlusion of the immobilized antibody molecules could be attributed to the low binding capacity for the target antigen. This target protein purification of 40 µg/ml of coupled gel was in accordance with purification using the same type of resin as described by Gallant et al. (2008).

The choice of detergent for extractions of membrane bound RVG is very important for the subsequent applications. The detergent used for solubilization should be easily removed by dialysis and should not interfere with further purification procedures. Earlier reports shows that purification is very complicated and needs additional steps such as isoelectric focusing when Triton X-100 was used for the extraction of RVG (Dietzchold et al., 1978). The protein mixture used for IAC was solubilized using CHAPS detergent which can be easily removed by
dialysis. The results confirm the suitability of CHAPS to solubilize the membrane bound rRVG since it does not interfere with subsequent purification steps. This is in agreement with the findings reported by Konig, (1972) that CHAPS detergent does not bind to ion-exchange resins and thus does not interfere with purification. Therefore, this IAC approach can be used for the purification of detergent solubilized RVG.

IAC for the purification of native RVG has been demonstrated earlier using a mAb raised against the synthetic tetrapeptide of G which is having the sequence similarity with the binding site of RV. This purification strategy was found to be a very sensitive, highly specific method for the purification of RVG without denaturing the protein Santucci et al. (1990). In the present study also, rRVG of considerable level of purity was obtained, as shown in the SDS-PAGE image (Figure 4.19) and the confirmation of purified rRVG was not altered when checked in mAb (M5B4) based IC-ELISA. Majority of the HCPs and host cell DNA were also removed from the complex mixture by IAC method of purification. MALDI-TOF analysis of the purified rRVG had shown the highest score hit for the G of PV strain of RV. The data obtained is in good agreement with Lai et al. (1981), confirming the identity of the material bound to the column at neutral pH and eluted at acidic pH (2.5) as RVG.

The rRVG with considerable purity with the single step of IAC is considered advantageous since the single step procedure avoids
excessive handling of samples and consequent losses. Using the mAb which binds to the trimeric form of the protein was helpful not only for accomplishing the tag-free purification but also for selectively enriching the immunogenic molecules from the mixture of various forms of rRVG. Also, this single step purification procedure could help to reduce the costs associated with the development of multiple downstream processes for the purification of tag free recombinant proteins.

5.5. Immunogenicity, protective efficacy and potency of BEVS expressed rRVG

The rRVG expressed in many different expression systems (Anaya et al., 2009; Klepfer et al., 1993; Prehaud et al., 1989; Tordo et al., 1993b; Tuchiya et al., 1992; Yelverton et al., 1983) met with variable success when evaluated for their immunogenicity and protective efficacy. Immunization of mice with insect cells expressing RVG induced protective neutralizing antibody titers (Prehaud et al., 1989). In raccoons, oral immunization with RVG containing insect cell lysate induced RVNA titers that protected most but not all of the animals from lethal challenge (Fu et al., 1993). Pseudo type baculovirus expressing the G of RV was found to be safe and immunogenic in mice (Huang et al., 2011). Since the use of cells or cell lysate as a vaccine may not be appropriate, an attempt was made to extract the membrane bound rRVG using several combinations of salts and detergents. Most of the RV neutralizing antibodies bind to conformation dependant epitope on the G and hence extracting the G
without altering its conformation is essential to preserve the immunogenicity. Thus the immunogenicity and protective efficacy of detergent solubilized rRVG was tested in mice. The mice were vaccinated intraperitoneally and challenged i.c with live RV on 35th DPI. All the mice vaccinated with solubilized rRVG had induced the protective RFFIT titer value whereas only 65 % - 67% of mice administered with the insoluble cell lysate had shown the protective titer value. In the challenge experiment, all the mice vaccinated with solubilized rRVG were protected even at the lowest dose of protein (0.2 µg) used in the experiment. These results confirm that the native conformation and immunogenicity of rRVG remained unaltered despite solubilization with CHAPS detergent. This confirms the statement that treatment using zwitterionic detergents is milder, less denaturing and maintains the biological activity of the protein. There are reports wherein rRVG expressing intact cells or crude cell lysate of insect cells was used for immunization (Fu et al., 1993; Prehaud et al., 1989). Prehaud et al. (1989) demonstrated that mice vaccinated with 12 to 120 µg of intact insect cell expressing RVG (10^6 to 10^7 whole insect cells expressing RVG) survived the peripheral challenge whereas in the present study even 0.2 µg of the solubilized insect cell expressed rRVG had shown 100% protection upon i.c challenge of RV. The protection conferred with lowest dose (0.2 µg) of solubilized rRVG was associated with the induction of RV neutralizing antibodies. This could be possible because of the use of solubilized preparation for
immunization which allowed quantifying the actual G content without having the interference of cellular proteins.

The international requirement for approval of inactivated rabies vaccine is to guarantee the quality of vaccines before releasing the batch. NIH potency test in mice remains the officially recommended test for evaluating the protective potential of commercial vaccines developed for use in all target species (Indian pharmacopoeia, 2007; WHO, 1992). We have adapted the NIH test procedure to see the potency of the detergent solubilized insect cell expressed rRVG based subunit vaccines. This test involves immunization on day 0 and 7 with various fivefold dilutions of IRS (ranging from 1/125 to 1/3125) and detergent solubilized rRVG (ranging from 1/5 to 1/3125) followed by i.c challenge of the immunized mice on day 14 with a standardized dose of the CVS strain of RV. As per WHO recommendation, the inactivated veterinary vaccines should have a minimum potency of 1 IU/dose and the modern rabies vaccines for human use should have a minimum potency of 2.5 IU/dose (WHO, 1992). The BEVS expressed rRVG has given potency around 0.7±0.15 IU per ~25 µg of solubilized G by NIH potency test. Thus ~36 µg of solubilized G may be sufficient to obtain a potency of approximately 1 IU/dose (for veterinary use). Plant expressed edible G based subunit vaccine was found to be effective at 50 µg of G per dose. All the mice immunized with 50 µg of G were protected against the virulent challenge (Loza-Rubio et al., 2008). From this study it is clear that ~36 to 50 µg of recombinant G
could be sufficient to give better protection. Based on the data obtained from the present study it is possible to formulate the recombinant G based subunit rabies vaccines for veterinary use.

In addition, the immunogenicity and protective efficacy of the rRVG after IAC purification was also checked in mice. The mice were immunized intraperitoneally twice with rRVG and then challenged i.c with CVS strain of RV. The results of RFFIT and challenge experiments have also confirmed that the immunogenicity of the rRVG was not altered after the purification. Eighty three percentage of the immunized mice survived the i/c challenge on 28 DPI. While 100% of the mice immunized with the solubilized rRVG was protected with the same antigen dose on day 35 DPI (Figure 4.23). Comparatively lesser percentage of protection for the same dose of rRVG could possibly due to the difference in the interval between immunization and challenge. The same kind of phenomena has been reported earlier by Peter et al., (2003) wherein the RVNA response and protection from i.c. challenge elicited by rabies vaccines in mice vary with the number of doses, route of administration, and time of challenge. However the rRVG was found to be immunogenic and protective after IAC purification also.