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# Immuno-affinity Purification of Insect Cell Expressed Rabies Virus Glycoprotein using a Conformational Specific Monoclonal Antibody

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**Abstract**

.Rabies is a disease of nervous system and causes progressive encephalitis with fatal outcome. The conformation-dependent epitopes on the glycoprotein (G) of rabies virus (RV) is responsible for the induction of virus neutralizing antibodies which is ultimately required to get complete protection from viral challenge. Therefore, a suitable chromatography technique is necessary to purify the tag free recombinant rabies virus glycoprotein (rRVG) without altering its immunogenic epitopes. The present study was undertaken to purify the rRVG using a conformational specific anti-rabies virus glycoprotein (RVG) mAb, M5B4, which binds to the natively folded G. The mAb had shown a significant kinetic interaction with RVG. The mAb immobilized onto the NHS-activated Sepharose 4 fast flow™ was used for the purification of rRVG by immuno-affinity chromatography (IAC). The bound rRVG was eluted in IAC using 0.1M glycine with pH 2.5 and the identity of the purified protein was confirmed by MALDI-TOF. The IAC purified rRVG induced neutralizing antibody response and 83% of the immunized mice were protected against intra-cerebral rabies virus challenge. The results indicate that the mAb based IAC method can be an effective purification technique for tag free rRVG with significant level of purity, without compromising the protein's immunogenic potential.

*Keywords:*Immuno-affinity purification; Rabies glycoprotein; insect cell; protective efficacy.

## Introduction

Rabies is one of the major zoonotic diseases and still an important public health problem in developing countries. The disease is caused by rabies virus (RV) and characterized by acute progressive encephalitis. The envelop glycoprotein (G) of RV, is the major antigen responsible for viral pathogenecity and is the only target for neutralizing antibodies (Dietzchold *et al*., 1978; Badrane and Tordo 2001). The advances in recombinant technology had led to the development of subunit and DNA vaccines based on rabies virus glycoprotein (RVG) (Yelverton *et al*., 1983; Fu *et al*., 1993; Xiang et al., 1994; Sakamoto et al., 1999; Biswas *et al*., 2001; Gupta *et al*., 2005; Ramya *et al*., 2011). The prime immune correlate of protection in case of rabies virus infection is induction of neutralizing antibodies and most of them bind to conformation dependant epitopes on the RVG (Ertl, 2009). Thus, purification of RVG without meddling the conformation-dependent epitopes is necessary to get complete protection to viral challenge. Though, there are many chromatography methodologies available, purification of RVG is a cumbersome, often unsuccessful and costly task.

The immuno-affinity chromatography (IAC) involves the immobilization of biological or synthetic ligands to inert resin and the resulting biospecific adsorbents will have high affinity for a single compound. The IAC technique using monoclonal antibodies (mAbs) are suitable for the purification of proteins from a variety of biological substances (Santucci *et al*., 1990). The recombinant proteins intended for vaccination purpose should not contain any affinity tag and thus, the IAC method can be a suitable alternative for the purification of tag-free recombinant RVG (rRVG) to be used as a vaccine candidate. Santucci *et al*. (1990) has reported that IAC using antipeptide antibodies as a highly specific and sensitive method for the purification

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of RVG.

In the present study, purification of the insect cell expressed rRVG was evaluated using a RVG site III specific mAb immobilized in an IAC. Immunogenicity and protective efficacy of the IAC purified rRVG was evaluated in mice.

## Materials and methods

*Cells, viruses, antibodies and animals*

Vero cell derived, beta propiolactone (BPL) inactivated rabies whole virus antigen (PV strain), obtained from the Human Biologicals Institute; Udhagamandalam, India was used to isolate the native RVG. The Challenge virus standard (CVS) obtained from Federal Vaccine Institute; Basel, Switzerland was used in mice challenge experiment. CVS 11 strain of RV procured from Agence Francaise De Securite Sanitaire Des Aliments, France and Neuro-2a cells obtained from ATCC, USA were used in Rapid fluorescent focus inhibition test (RFFIT) to determine the neutralizing antibody response. A *Spodoptera frugiperda* (Sf9) cell obtained from Ingenasa, Spain was used for the production of rRVG. RVG site III specific mAb, M5B4 (Nagarajan *et al*., 2006) was used in IAC and ELISA.

Swiss albino mice (12-15 g body weight) of both sex, procured from National Institute of Nutrition (NIN), Hyderabad, India was used for assessing the immunogenicity and protective efficacy. The challenge experiments were carried out in Biosafety level II at small animal testing facility, Indian Immunologicals Limited (IIL), Hyderabad, India following the guidelines of Institutional animal ethical committee.

*Measurement of affinity constant of mAb-M5B4 by BIAcore*

The affinity constant of mAb (M5B4) with Native RVG was measured at room temperature (23 to 25°C) using BIAcore 3000 SPR biosensor (GE, Sweden) as described previously (Kim *et al*., 2003; 2006). Briefly, the carboxymethylated dextran matrix (CM-5 chip) was activated with a mixture of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC; 0.05M) and N-hydroxysuccinimide (NHS; 0.2 M). RVG was immobilized onto the activated dextran surface using 10mM sodium acetate at pH 4-5.5 with a flow rate of 10 µl/min. The mAb (M5B4) was diluted in PBS to final concentrations of 2 nM, 4 nM, 6nM, 8 nM and 1µM and was injected individually at a flow rate of 30 µl/minute (Gil *et al*., 2002). An unrelated antigen (Yeast expressed HBsAg) was immobilized onto the control channel under the same conditions to normalize the instrument and buffer artifacts. The dissociation and association rate constants were obtained by statistical analysis of the data using the BIAcore Evaluation software (BIAevaluation version 4.1) provided by the manufacturer.

*Preparation of immune-affinity resin*

Coupling of mAb with resin

The mAb (M5B4) was immobilized onto NHS-activated Sepharose 4 fast flow TM (GE Healthcare, Sweden) following the procedure recommended by manufacturer with minor modifications (Gallant *et al*., 2008). Briefly, the mAb was dialysed against coupling buffer (0.2M sodium bicarbonate, 0.5M NaCl, pH 8.3); covalently coupled with the resin by gentle end-to-end mixing at 25°C for 4 hours and the coupled matrix was packed in an empty column. The coupling efficiency (δ) was determined by an indirect method as described by Hernandez *et al.* (2001) following the formula; $\delta \% = \chi / \lambda \times 100$, where χ is the amount of coupled protein determined as the difference between the original amount of ligand or mAb and δ is the amount detected in the washing fractions after coupling. The packed column was stored at 2-8°C in 20% ethanol until further use.

Evaluation of ligand leakage

The amount of ligand leakage or the coupling density was measured by an antibody mediated ELISA (Subramanian *et al*., 1994). Polystyrene plates were coated with the purified antigen from PV strain of RV (500 ng/well) for overnight at 2-8°C. The plate was washed and blocked with 1% bovine gelatin in PBST and incubated at 37°C for 1 hour. The plate was washed again and the wash fractions from the coupled matrix (which may contain the uncoupled mAb) was added. The plate was incubated at 37°C for 2 hours and following subsequent washing, goat anti-mouse IgG-HRPO was added. The reaction was developed using a substrate acti-

vated chromogen (TMB) and the colour development was measured in an ELISA reader at 450 nm wave length.

*rRVG for Immuno-affinity purification*

The rRVG was expressed in Sf9 cells and the quantity of rRVG present in the soluble fraction was measured using an immunocapture (IC)-ELISA as described earlier (Ramya *et al*., 2011). Briefly, Sf9 cells were infected with the recombinant baculovirus having the RVG expression cassette at a MOI of 4. The cells were harvested 72 hrs post infection and washed thrice with PBS. The cell pellet was lysed using the lysis buffer, formulated with 50mM Tris-HCl, 150mM NaCl, 4mM EDTA (pH 7.4), 10% DMSO and 0.6% CHAPS detergent. The mixture was then incubated for 30 min by end-to-end constant mixing at RT (23 to 25$^oC$). Finally, the lysate was clarified, filtered and dialyzed against PBS (pH 7.2).

*Optimization of elution buffer conditions for IAC by ELISA*

Elution buffer for the optimal recovery of bound rRVG was determined using an ELISA method (Santucci *et al*., 1990; Ibarra *et al*., 1999; Ramya *et al*., 2011). The microtitre plates were coated with a known concentration of unlabelled mAb-M5B4 and incubated overnight at 2-8$^oC$. The wells were washed thrice with PBST and blocked for 1 hour at 37$^oC$ using 1% (w/v) bovine gelatin in PBST. The solubilized rRVG was added to the wells and subjected to serial two fold dilutions. The in-house reference standard (IRS) with known G content was also included in the ELISA. The plate was incubated for 1 hour at 37$^oC$. The wells were washed thrice with PBST and incubated with various elution reagents: a) 0.1M glycine-HCl, pH 2.5; b) 0.2M glycine-HCl, pH 3.5; c) 0.1M sodium bicarbonate, pH 8.0 d) 8M urea, pH 7.0 and e) PBS, pH 7.0 (negative control) for 15 minutes at 37$^oC$. The wells were washed thrice with PBST and incubated with the biotinylated mAb- M5B4 for 1 hour at 37$^oC$. This was followed by the addition of streptavidin-peroxidase conjugate (Sigma, USA) and incubated for 1 hour at 37$^oC$. The enzymatic color reaction was developed using a chromogen solution and the absorbance was read in an ELISA reader at 450 nm wave length. The bound un-eluted RVG from various treatment groups was quantified using a standard curve obtained for IRS (Nagarajan *et al*., 2006). The amount of RVG recovered after the buffer treatment was estimated by comparing the bound RVG quantity in the negative control (PBS). Buffer which had shown a higher percentage of recovery of the bound protein was chosen as elution buffer for IAC (Agraz *et al*., 1994; Ibarra *et al*., 1999).

*Optimization of immuno-affinity chromatography*

The cell lysate was passed through the immunoadsorbent matrix at a flow rate of 1 ml/minute followed by a residence time of 30-60 minutes. The sample was applied twice and the unbound proteins were collected in the flow through. The column was washed with 5-10 column volumes of PBS at a flow rate of 2 ml/minute. The bound rRVG was eluted with 0.1M glycine, pH 2.5 at a flow rate of 1 ml/minute and the elutes were neutralized with 1/10th the volume of 1M Tris-HCl, pH 8.6. The column was subsequently washed with 10 column volumes of PBS and stored in 20% ethanol at 2-8$^oC$ until further use (Gallent *et al*., 2008).

*Analysis of the immuno-affinity purified rRVG*

The elution fractions from IAC were assayed for rRVG quantity by IC-ELISA and the peak elutes with maximum antigen content were pooled. The rRVG in the pooled elutes was quantified using IC-ELISA. Purity of the protein was analyzed by SDS-PAGE. The ligand leakage during elution was also analyzed by antibody mediated ELISA as described earlier.

*Immunogenicity and protective efficacy of IAC purified rRVG*

The immunogenic property of IAC purified rRVG was analyzed in Swiss albino mice (12 to 15 g body weight). The mice were immunized with 0.4 µg of algel adjuvanted IAC purified rRVG by intraperitoneal route twice on day 0 and day 14. Mice in the control group were inoculated with a placebo formulation.

Seven days post booster immunization, the sera were collected and RV neutralizing antibody (RVNA) titers were determined by RFFIT (Smith *et al*., 1996). Briefly, various dilutions of test and

reference sera (Standard rabies immunoglobulin; SRIG, NIBSC, UK) were mixed with 50 $FFD_{50}$ of CVS-11 strain of RV and incubated at 37ºC in the presence of 5% $CO_2$ for 90 minutes. After the incubation period, Neuro-2a cells were added to the mixture and incubated again for 20 hours at 37ºC with 5% $CO_2$. The cell sheet was then fixed with acetone and stained using anti-RV nucleocapsid mAb conjugated with FITC (Chemicon, USA). Based on the presence of unneutralized virus across various dilutions, the titer was expressed as the reciprocal of the dilution that neutralizes 50% of the added virus. The antibody titers of the sample sera were expressed as IU/ml in comparison with the 50% end point titer obtained for the NIBSC standard serum.

The protective efficacy of the purified rRVG was analyzed by challenging the experimental mice intracerebrally (i/c) on day 28 post immunization with $30LD_{50}$ dose of CVS strain of RV. Mice were observed for the development of symptoms typical of rabies on a daily basis for 14 days post challenge. Death of mice in the first 48 hrs post inoculation was considered non-specific and those mice were eliminated from the study. Finally, the percentage protection was calculated for all the experimental groups.

## Results

*BIAcore analysis*

The mAb binding to the natively folded RVG at antigenic site III (M5B4) was used in the preparation of immuno-adsorbent to purify rRVG. The affinity constant of the mAb was evaluated using BIAcore immunoassay and the mAb had a KD value of 2.06 x $10^{-7}$ M, indicating a significant kinetic interaction with the antigen at nanomolar concentrations (Fig. 1).

*Immuno-affinity purification of rRVG*

The mAb was immobilized at a protein concentration of 10 mg/ml on NHS-activated Sepharose 4 fast flow™ at pH 8.3. The amount of uncoupled antibody (post-coupling and blocking) and leaching of the coupled mAb during the purification process were measured using an antibody mediated ELISA. Around 93 to 95% of the antibody was coupled to the activated matrix and leaching of the mAb in all column washes and elutes as measured by ELISA was minimal.

The elution buffer composition was chosen from a wide range of buffers and their efficiency was analyzed by quantifying the unrecovered bound rRVG using an IC- ELISA. The result had shown that 0.1M glycine (pH 2.5) and 0.2M glycine (pH 3.5) has eluted around 80% and 40% of the bound rRVG, respectively. While, the 0.1M sodium bicarbonate (pH 8.0) could elute only 10% of the bound rRVG. The wells added with 8M urea showed very low ELISA ODs as the urea might denature the bound rRVG (Fig. 2). Ten different purification runs with varying concentration of rRVG content were performed with three different bed volumes of coupled gel to evaluate the consistency in the performance. On an average, a recovery rate of >90% was obtained with the dynamic binding capacity of the resin being 40µg of rRVG per ml of

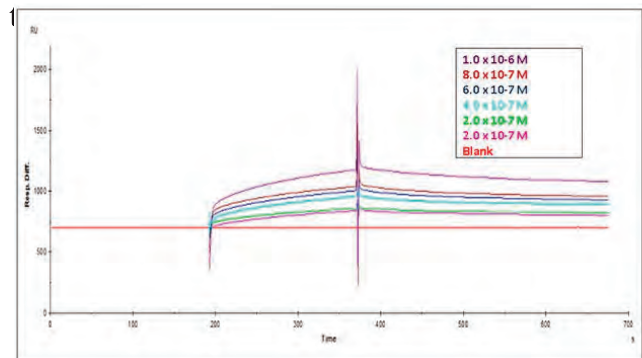


Fig. 1. Sensorgram of BIAcore assay showing kinetic binding curves of RVG with different concentrations of mAb-M5B4 (2 nM, 4 nM, 6nM, 8 nM and 1µM) indicated by different color. The mAb had shown an affinity constant of 2.06 x $10^{-7}$ M at equilibrium.

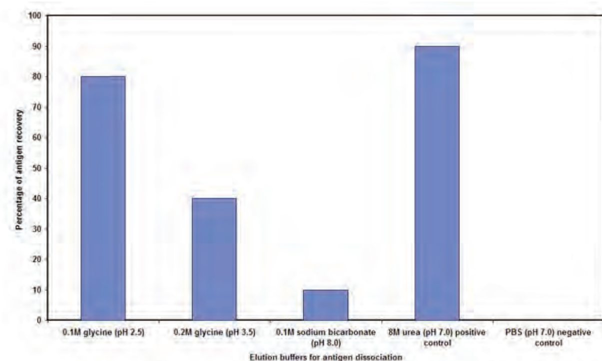


Fig. 2. rRVG recovery with different elution buffers as demonstrated by IC-ELISA. The rRVG was captured on the ELISA plates using mAb, M5B4 and various elution buffers were tested for their ability to elute the bound antigen. Percentage recovery of bound rRVG was calculated based on the reduction in rRVG quantity compared to the PBS control. The result showed that 80% of the bound rRVG was eluted with 0.1 M glycine (pH 2.5). The 8M urea (pH 7.0) had completely abrogated the ELISA activity due to its denaturing property.

*Analysis of the immunoaffinity purified rRVG*

The eluted protein was neutralized by a suitable buffer of alkaline pH to avoid acid-induced protein denaturation and the specific reactivity of the neutralized rRVG was verified using IC-ELISA. The IC-ELISA results indicated that the purification strategy has not altered the conformation of rRVG. When the IAC purified rRVG was analyzed using SDS-PAGE, a protein band of ~55 kDa, corresponding to the size of RVG was observed (Fig. 3). The protein band was subjected to MALDI-TOF and the identity of protein was confirmed as the G protein of PV strain of RV. Thus, the RVG bound to the column at neutral pH and eluted at pH 2.5. These results are in agreement with the previous reports (Lai, 1981; Santucci *et al.*, 1990).

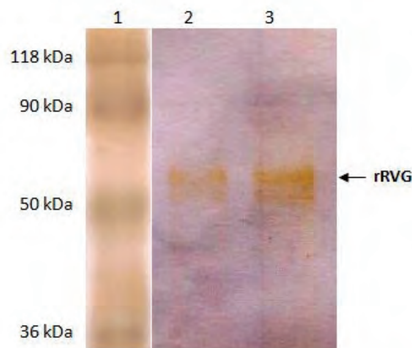


Fig. 3. Purification of rRVG by immunoaffinity chromatography using mAb-M5B4 coupled with NHS-activated Sepharose. The purified rRVG was analyzed using 12% reducing SDS-PAGE and stained with silver nitrate. Lane 1- Molecular weight marker. Lane 2 and 3 are showing the ~55kDa band of rRVG post purification.

*Immunogenicity and protective efficacy of the IAC purified rRVG*

The immunogenicity of IAC purified rRVG was assessed by inoculating the mice intraperitoneally on days 0 and 14 with the immuno-affinity purified rRVG. RVNA titers were measured from the sera collected on day 21 post immunization and the percentage of mice showing more than 0.5 IU/ml (minimum protective RFFIT titer as recommended by WHO) was calculated. Around 67% of mice inoculated with the IAC purified rRVG exhibited protective titer on day 21 post vaccination with an average titer of 1.46 ±1.4 (mean ± SD) and 83% of immunized mice were protected against the intracerebral challenge performed on day 28 post immunization. All the unimmunized mice developed rabies specific symptoms and died.

## Discussion

The available rabies vaccines contain inactivated rabies virus and these vaccines are produced by growing the virus in large scale using primary or continuous cell lines. Therefore, very strict bio-security measures are followed in the vaccine manufacturing facilities. Creating and maintaining the bio-security procedures involve huge expenditure. Personnel working in the manufacturing facility of cell culture based rabies vaccines are also at risk for the infection (Winkler *et al.*, 1973). A recombinant sub-unit vaccine could be an alternative to avoid the handling of live rabies virus in the vaccine manufacturing facilities. RVG alone can induce neutralizing antibody response against rabies virus and the sub-unit vaccines developed using RVG had been found successful in inducing neutralizing antibody response and protecting mice against intra-cerebral mice challenge (Ertl, 2009; Ramya *et al.*, 2011). The RVG is present in trimeric form and expressing the RVG with transmembrane domain (TMD) is essential to maintain its trimeric structure (Desmezieres *et al.*, 2003). As many of the RV neutralizing epitopes are conformation dependent, the membrane extraction had to be performed carefully to protect the immunogenic property of the G.

The recombinant proteins intended for animal or human vaccination should be devoid of any affinity tag to meet the regulatory requirements. Hence, a suitable method is required for the purification of membrane bound and affinity tag free RVG without altering its immunogenic conformation. 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.

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 X $10^{-7}$ M was obtained for the mAb at equilibrium, making it suitable for IAC. An ideal elution buffer for IAC

should effectively release the protein without abrogating the function of the protein. Among the elution buffers tested, the 0.1M glycine with pH 2.5 could elute 80% of bound proteins. The use of low pH elution buffer does not affect the binding activity of the mAb, M5B4, when the elutes are verified in IC-ELISA and this result is in agreement with the earlier report (Santucci *et al.*, 1990).

Santucci *et al*. (1990) had demonstrated that the IAC based purification of RVG using a mAb raised against the synthetic tetrapeptide of G. This purification strategy was found to be a very sensitive, highly specific method for the purification of RVG without denaturing the protein. In the present study also, rRVG of considerable level of purity was obtained, as shown in the SDS-PAGE image (Fig 3) and the conformation of purified rRVG was not altered when checked in mAb (M5B4) based IC-ELISA . In addition, the results of in-vivo mice experiments have also confirmed that the immunogenicity of the rRVG was not altered after the purification and 83% of the immunized mice survived the i/c challenge. 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.

Taken together, these results indicate that the mAb based IAC could be a dependable technique for the purification of RVG without altering its native conformation and the immunogenic property.

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