Journal of Chromatography B, 876 (2008) 109–115

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Chromatographic purification of equine immunoglobulin G F(ab)$_2$ from plasma

Aldon Fernandes[a], John Oswald Kaundinya[b], Gautam Daftary[a], Lalit Saxena[c], Subhasis Banerjee[c], Priyabrata Pattnaik[c,*]

[a] Bharat Serum and Vaccines Ltd., Plot No. A-371-372, Road 27, Wagle Industrial Estate, Thane 400604, India
[b] BSV BioSciences, Inc., 875 Jarvis Drive, Suite 140, Morgan Hill, CA 95037, USA
[c] Millipore India Pvt. Ltd., Bioprocess Division, 50A, 2nd Phase, Ring Road, Peenya, Bangalore 560058, India

ARTICLE INFO

Article history:
Received 25 May 2008
Accepted 17 October 2008
Available online 1 November 2008

Keywords:
Plasma fractionation
Equine
F(ab)$_2$
Plasma IgG
Purification
Chromatography
Enzymatic digestion
ProSep-vA Ultra
Cellufine A-200

ABSTRACT

The antibody fragments generated from hyperimmune equine IgG is widely used as anti-snake venom, anti-scorpion venom, anti-diphtheria, anti-tetanus, anti-gangrene and anti-rabies agents. Antibody fragments, F(ab)$_2$, because of their specificity and absence of undesired reactivity are preferred over complete IgG. This paper discusses a novel purification technique for chromatographic purification of anti-rabies immunoglobulin G (IgG) fragment F(ab)$_2$ from horse serum. F(ab)$_2$ was purified by two successive chromatography steps using Cellufine A-200 and ProSep-vA Ultra media. The purified F(ab)$_2$ was characterized using biochemical and biophysical methods and shown to be pure and homogeneous. The purified F(ab)$_2$ was reactive to rabies antigen in immuno-electrophoresis and diffusion tests. The purified F(ab)$_2$ was biologically functional and was found to show a potency of 1500 IU ml^{-1}. Comparative analysis of the purity with commercially available F(ab)$_2$ by HPLC analysis and SDS–PAGE indicated that the present product is better in purity. To our knowledge, this is the first report providing evidence on purification of equine antibody fragment using controlled pore glass based protein A chromatography media.

1. Introduction

Antibody-based therapeutics using polyclonal or monoclonal antibodies are increasingly being developed for use in indications such as oncology, inflammation, and infectious diseases. Advances in antibody technologies have facilitated the rapid generation of high-affinity antibodies of defined specificity and have led to the development of a diverse range of antibody-based molecules formatted to address specific applications [1]. High dose requirements for treatment of chronic indications in large markets necessitate the development of a high yielding, low-cost manufacturing process that consistently delivers high-quality product [2]. With increasing emphasis on new technologies with monoclonal antibody expression and purification, the clinical need of polyclonal therapeutics for a variety of specific illnesses and infections is often overlooked. Polyclonal antibody therapeutics are today widely used in medicines for viral and toxin neutralization and for replacement therapy in patients with immunoglobulin deficiencies [3]. Over the past 20 years, intravenous immunoglobulins have shown beneficial immunomodulatory and anti-inflammatory effects in such illnesses [4].

Hyperimmune antibody preparations produced from horse serum have been used over the past century for the treatment of a variety of infectious agents and medical emergencies, including dioxin toxicity, snake envenomation and spider bites [5–7]. Equine IgG is also used for the treatment of rabies [8] and tetanus [9]. Any clinically used antibody needs to be highly pure since contaminating serum proteins may elicit various adverse reactions in patients [10]. Equine IgG is extensively used in developing countries where product cost can become a major limiting factor [11–13]. Theakston and Warrell [14,15] and Theakston et al. [7] in their review emphasized the challenges faced by developing countries on availability, manufacture and distribution of hyperimmune serum and immunoglobulins. The purification of equine IgG is therefore challenging since the product has to be extensively purified in quite large quantities in an economical way from horse serum.

Traditionally, equine IgG has been purified by ammonium sulphate induced precipitation [16] or by caprylic acid-based fractionation [16]. The limitations of ammonium sulphate induced precipitation are discussed in the next paragraph. Caprylic acid-based fractionation which relies on selective precipitation of all non-IgG serum protein is also widely used for manufacturing antivenom and similar immunological products. The IgG purity

* Corresponding author. Tel.: +91 80 39282452; fax: +91 80 28396345.
E-mail addresses: p_pattnaik@millipore.com, p.pat@rediffmail.com (P. Pattnaik).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.10.030

obtained from a single stage caprylic acid precipitation process is about 70% and recovery is about 67% [16]. Purification of equine IgG by ion-exchange chromatography [17], hydrophobic interaction chromatography [18] and protein A or protein G-based affinity chromatography [19] has been reported. However, chromatography as a unit operation is considered scalable and the major limitation is therefore cost of operation. Hence commercial equine IgG production using these methods has not been feasible. The bottom line is that any hyperimmune equine IgG purification technique will have to be simple and economical yet effective in meeting purity requirements.

Ammonium sulphate induced precipitation followed by centrifugation is widely used for manufacturing equine IgG for antivenom applications. However, this technique suffers from both low yield and low purity. The basis for separation is the preferential precipitation of IgG at high ammonium sulphate concentration, typically in the 1.6–2.0 M range. At lower salt concentrations within this range the precipitate is primarily composed of IgG (i.e., purity is reasonable) but a substantial amount of the antibody still remains in solution and therefore the recovery is very low. On the other hand, at higher salt concentrations, recovery of IgG is very high but substantial amounts of impurities, primarily serum albumin, are also precipitated leading to very low purity. Another key issue with ammonium sulphate induced precipitation-centrifugation and indeed all types of conventional precipitation-centrifugation processes are the entrapment of impure molecules within the precipitated particles and in some cases co-precipitation.

The limitations of ammonium sulphate induced precipitation-centrifugation for purification of $F(ab)_2$ can be overcome by using a hybrid bioseparation technique, which combines enzyme treatment, precipitation and high-resolution chromatography. The use of hybrid bioseparation has been reported for the purification of human IgG [20] and humanized monoclonal IgG [21]. It was generally accepted that the removal of Fc fragments from IgG prevented complement activation and so reduced the risk of reactions. However, although Fc is not required for antigen neutralization, it was suggested that it might be involved in Ag–Ab complex elimination. More controlled clinical trials are necessary to characterize the efficacy and safety of these products [7].

Use of antibody fragments [$F(ab_2$] in place of whole antibody is preferred due to favorable pharmacokinetic profile, i.e., their wide volume distribution and ability to reach tissue compartment at a faster rate when compared to IgG due to low molecular weight and less immunogenicity [22,23]. Efficient separation of active $F(ab)_2$ from Fc fragments and other serum proteins has remained a challenge. Earlier methods involved ammonium sulphate fractionation, heat treatment and combination of the two [15,24,25]. It has been claimed that ammonium sulphate fractionation is time consuming, costly and produces a mixture of $F(ab)_2$ and uncleaved IgG [3]. Later caprylic acid-based purification method was developed which proved to be better in terms of production time, yield, albumin contaminants and turbidity [26]. To date, chromatography remains the most commonly employed method for the purification of IgG digest. Several different techniques like protein A affinity-, immobilized metal affinity-, ion exchange-, hydroxyapatite-, hydrophobic interaction-, hydrophobic charge induction-, and size exclusion chromatography have been employed [27–29]. Despite many references available on the purification of IgG fragment, it still remains a challenge to purify $F(ab)_2$ fragments at the industrial scale.

In this paper we explain an innovative method of purifying equine $F(ab)_2$ fragment from horse serum by using a combination of ion-exchange and protein A based affinity purification which may be a linearly scalable option of industrial-scale production of $F(ab)_2$ fragments.

2. Experimental

2.1. Instruments

The chromatographic system used throughout this study was the fast protein liquid chromatography (FPLC) system; model AKTA-Prime from Amersham Biosciences AB (Uppsala, Sweden). Cooling centrifuge, model compufuge CPR-30 was from Remi (Mumbai, India). Spectrophotometer UV-1 NC-9423 was from Thermo Spectronic (Waltham, MA, USA). The Pellicon XL tangential flow filtration system was from Millipore Corporation (Bedford, MA, USA). The electrophoresis apparatus X cell surelock Mini-Cell was from Invitrogen Corporation (Carlsbad, CA, USA). HPLC system LC–2010^{HT} was from Shimadzu (Nakagyo-ku Kyoto, Japan). Immuno-electrophoresis apparatus Model sub cell-192 cell was from Genei (Bangalore, India).

2.2. Materials and chemicals

Goat anti-horse whole serum GWS-70 was from Immunology Consultant Lab (Newberg, OR, USA). Cellufine A-200 was from Chisso Corporation (Chuo-Ku, Tokyo, Japan). Posep vA Ultra, Vantage L column 44/500 mm, 22/500 mm and Pellicon XL Biomax-50 kDa Ultrafiltration devices were from Millipore Corporation (Bedford, MA, USA). Sodium chloride salt, sodium acetate, HCl, Cresol chemicals, all reagents for electrophoresis such as SDS, polyacrylamide, Coomassie brilliant blue R250 and pepsin enzyme (EC: 3.4.23.1) were from Sigma Chemical (St. Louis, MO, USA). TSK-G3000 SWXL HPLC column was from TosoHass (Stuttgart, Germany). HPLC grade sodium phosphate dihydrate AR and Disodium phosphate hydrate AR were from Fluka (Buchs/Switzerland). Caprylic acid was from Alfa Aesar Johnson Matthey Corporation (MA, USA). Molecular weight marker for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was from Genei (Bangalore, India).

2.3. Preparation $F(ab)_2$ from plasma

2.3.1. Immunization of horse

Commercially available human rabies vaccine (Rabipur) was used as antigen to immunize horses. Horses weighing 250–350 kg and ranging from 5 to 10 years of age were immunized intramuscularly with rabies vaccine (2.5 IU ml^{-1}). A total of 30 human doses of rabies vaccines were injected to horses in a span of 2 weeks. Plasma was collected from horses that had been immunized with gradually increasing dose after every 4th day [30,31]. A total of 104 animals were immunized.

2.3.2. Collection of hyperimmune plasma

The hyperimmunized animals were maintained as per the guidelines of CPCSEA (Committee for Purpose of Controlled and Supervision of Experiment on Animals), India. The healthy animals (horses) were immunized at an interval of every 3 days for 2 weeks and the antibody titer in blood was estimated by ELISA [32–35]. After 2 weeks, 10 ml of hyperimmune plasma was collected (test bleed) from each immunized animals and antibody titer value (potency) in plasma was estimated by rabies antigen neutralization using Swiss albino mice [36–38]. Based on the results of test bleed, animals were bled and hyperimmunised plasma was collected in sterilized nonpyrogenic glass container containing anticoagulant acid citrate dextrose (ACD).

The average anti-rabies potency of hyperimmune plasma was measured by antigen neutralization.

2.3.3. Antiserum manufacturing process

2.3.3.1. Pepsin activity. The enzymatic activity of pepsin was determined by the procedure described by Ryle [39] using hemoglobin as substrate. Pepsin digestion of horse plasma was carried out using pepsin EC: 3.4.23.1. One unit is defined as quantity of enzyme required to obtain 0.001 absorbance of soluble peptide derived from Ryles assay [39].

2.3.3.2. Enzymatic digestion of defibrinated plasma. The Equine serum was brought to room temperature (25 °C) and was diluted from 80 to 40 mg ml^{-1} with cooled water for injections (WFI). The pH was adjusted to 3.5 ± 0.1 with 1N HCl and the temperature to 37 ± 2 °C. Pepsin EC: 3.4.23.1 was added to give an enzyme substrate ratio of 1:50 with slow stirring conditions for 240 min at 37 ± 2 °C. The digestion was stopped by adding 1N NaOH base to bring the pH to 5.0.

2.3.3.3. Octanoic acid precipitation. The digested hyperimmune sera was incubated for 20 min at 56 ± 1 °C. The sera was then centrifuged at 10,000 × *g* for 30 min to remove precipitated impurities including fibrinogen at 25 ± 2 °C. Octanoic acid was added slowly to give final concentration of 6% with vigorous stirring for 30 min at 25 °C and the mixtures were centrifuged at 10,000 × *g* for 30 min. The proteins in the supernatants were filtered using 2 μm followed by multilayer 0.45/0.22 μm filter (Millipore, USA) and subsequent supernatant obtained and diafiltered against 10 volume of buffer containing 0.85% sodium chloride saline solution using Pellicon XL 50 kDa Biomax cassette at 5 ± 0.1 °C. The F(ab)$_2$ thus obtained was stored at 4 °C until further analysis. The antibody activity was estimated by a bioassay procedure and 70% of protein was found to be recovered.

2.4. Chromatographic procedure-1

2.4.1. Preparation for the ion-exchange chromatography

The Cellufine A-200 resin was packed in a Vantage L (44 mm diameter) column. Slurry was prepared with 15 mM sodium acetate buffer + 50 mM NaCl, pH 5.7, in a ratio of 75% settled gel to 25% buffer. The column was filled through the outlet with a few centimeters of buffer and was closed. The gel slurry was poured into the column in one continuous motion. The remainder of the column was filled with buffer and the top piece mounted and connected to a pump. The bottom outlet of the column was opened and the pump was set at 27.4 ml min^{-1}. The packing flow-rate was maintained for three bed volumes after a constant bed height (26.3 cm) was reached. The column was washed with 10 column volumes of water for injection at a flow-rate of 27.4 ml min^{-1}. The column was equilibrated with 10 column volumes of 15 mM sodium acetate buffer, pH 5.7 containing 50 mM NaCl having 6 mS cm^{-1} conductivity.

2.4.2. Ion-exchange chromatography procedure

Pepsin digested and octanoic acid precipitated plasma was loaded to the pre-equilibrated Cellufine A-200 column at a linear velocity of 90 cm h^{-1} at 15–20 °C. Post-load was collected in fractions of 60 ml each. The column was washed with equilibration buffer at pH 5.7, at a linear velocity of 90 cm h^{-1} until UV baseline was reached. The bound protein was eluted using a step gradient of buffer at pH 5.7 with NaCl starting at conductivity 20 and 50 mS cm^{-1}. The elution buffer has similar composition and same pH as equilibration buffer, but salt concentration (conductivity) was different. The emergence of protein in the fractions was monitored by spectrophotometer by taking OD at 280 nm. 100 μl of each fraction was ethanol precipitated and analyzed by SDS–PAGE. The relevant fractions were pooled and diafiltered using 50 cm^2 TFF device to 20 mM sodium phosphate buffer at pH 7.3 (5.0 mS cm^{-1}) and final protein concentration was adjusted to 7.0 mg ml^{-1} processed for further purification.

2.5. Chromatographic procedure-2

2.5.1. Preparation for the protein A based affinity chromatography

The ProSep-vA Ultra resin was packed in a Vantage L (22 mm diameter) column. Slurry was prepared with 20 mM sodium phosphate buffer, pH 7.3, in a ratio of 60% settled matrix to 40% buffer. The column was filled through the outlet with a few centimeters of buffer and was closed. The gel slurry was poured into the column in one continuous motion. The remainder of the column was filled with buffer and the top piece mounted and connected to a pump. The bottom outlet of the column was opened and the pump was set at 19 ml min^{-1}. The packing flow-rate was maintained for five bed volumes. The column was gently tapped using a teflon rod for consolidation of bed till 21 cm bed height was reached. The final volume of the media was 80.0 ml. The column was washed with five column volumes of water for injection. The column was equilibrated with 10 column volumes of 10 mM sodium phosphate buffer, pH 7.3 containing 750 mM sodium sulphate at a conductivity of 70 mS cm^{-1}.

2.5.2. Protein A based affinity chromatography procedure

Post-load samples from the Cellufine A-200 column were loaded to the pre-equilibrated ProSep-vA Ultra column at a linear velocity of 253 cm h^{-1} at 15–20 °C by maintaining residence time of 5 min. The column was washed with equilibration buffer at a linear velocity flow rate of 253 cm h^{-1} until UV baseline was reached. The bound protein was eluted using a 100 mM sodium acetate buffer, pH 3.0. Total protein content in individual fractions of post-load (flow through) and elute was measured by spectrophotometry by taking OD at 280 nm. 100 μl of each fraction was ethanol precipitated and analyzed by SDS–PAGE. The relevant fractions were pooled, and concentrated using 50 cm^2 TFF device and adjusted to 20 mg ml^{-1} with equilibration buffer.

2.6. Analytical procedures

2.6.1. Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using X cell surelock Mini-Cell apparatus and Tris-glycine-SDS buffer (Lamaelli buffer) were used to monitor the purification during chromatographic procedures. Electrophoresis was performed for 90 min at 100 V using 10% polyacrylamide gels. Detection was performed with silver staining. Low Molecular weight marker (97.400–6.500 kDa) loaded in first well of gel to compare molecular weight.

2.6.2. Potency estimation

2.6.2.1. Potency estimation by bioassay. Ten-fold serial dilution of purified F(ab)$_2$ was done in normal saline. Mixture of given dilution of purified F(ab)$_2$ was mixed in 1:1 ration with of rabies antigen so as to get a final volume of 5.0 ml. The mixture was allowed to stand in water bath at 37 °C for 30 min and centrifuged to remove precipitate (if any). The 0.5 ml mixture was injected intravenously (I.V.) to a set of eight Swiss albino mice each weighing 18–20 g. The same experiment was carried out with all the dilutions of purified F(ab)$_2$ with similar sets of mice. The death/survival of mice was recorded after 48 h of injection. Survival curve was plotted for each dilutions of F(ab)$_2$ following standard static method.

2.6.2.2. Potency estimation by ELISA. Potency estimation was done by using Bio-Rad ELISA Kit. Kit Reagents were brought to room temperature prior to use. Dilutions ranging from 1:10 to 1:200,000 were done in sample diluent to be used as standard. 0.1 ml of standard (dilutions ranging from 1:30,000 to 1:200,000) and 0.1 ml of sample (dilutions 1:100,000 and 1:200,000) were loaded into each well of the precoated strips, and incubated for 60 min at 37 °C. After four washes with wash buffer from the kit, 0.1 ml of a 1:1000 diluted Rabbit anti-horse IgG peroxidase conjugate was loaded into each well, and incubated for 60 min at 37 °C. After washing, 0.1 ml of TMB substrate from the kit (1:10 dilution) was added to each well and incubated for 10 min. After desired color development, 0.1 ml of stop reagent was added. The absorbance was measured at a dual wavelength of 450–630 nm. A standard graph was plotted of titer (IU ml^{-1}) versus absorbance, and the titer of the unknown sample was extrapolated from the standard graph [32,33].

2.6.3. Gel permeation chromatography (HPLC)

The TSK Gel-3000 SWXL HPLC-SEC column was equilibrated with 20 mM phosphate buffer in 150 mM sodium chloride solution, pH 7.2 at a constant flow-rate of 1.0 ml min^{-1}. Retention time of different standard proteins in the column at a flow-rate of 1 ml min^{-1} was standardized using LMW gel filtration calibration kit. Purified $F(ab)_2$ was loaded (20 μl) to the pre-equilibrated and pre-calibrated TSK Gel-3000 SWXL HPLC column. Elution of purified $F(ab)_2$ from the column was monitored at 280 nm and elution time was compared with the standard proteins.

2.6.4. Immuno-precipitation

Immuno-electrophoresis was carried out in a 1% agarose gel. 300 μg solution of purified $F(ab)_2$ was loaded in the well and protein was allowed to separate along the gel slide. Immuno-electrophoresis was carried out in sodium barbitone buffer (0.416%), pH 8.6 at 20 mA until the control dye reached the end-plate. Wells were cut in the gel and 150 μl of anti-horse serum was loaded to the wells. The plate was maintained in a moist chamber overnight at room temperature. At the end of 24 h, the plates were dried on a gelatin sheet and stained using Coomassie brilliant R250 and observed for appearance of precipitations arch. Commercially available $F(ab)_2$, i.e., Pasteur $F(ab)_2$ and equine rabies antiserum B. No. 212 was used as control samples. Different fractions collected at different stages of purification of $F(ab)_2$ was also used evaluate purity and reactivity. Single arc in the gel considered as desired purity and specificity and more numbers of arcs signify high impurities.

3. Results and discussion

3.1. Enzymatic digestion and acid precipitation

Horse hyperimmune serum and plasma against rabies was used as test material. A preliminary experiment was carried out to study octanoic acid (OA) fractional precipitation of horse serum proteins, using 1–8% of the organic acid. Most serum proteins were precipitated at OA concentration of 2%, no additional proteins were precipitated at concentrations higher than 6%. Protein analysis by SDS–PAGE showed that albumin was completely precipitated by 2% OA. OA, at 1–2% concentration, did not precipitate IgG while some IgG was precipitated at 3–6% concentration (data not shown).

When hyperimmune antirabies serum digested plasma was fractionated by OA, most proteins were precipitated at an OA concentration of 6%. OA concentration higher than 6% rendered supernatant with excessive turbidity and relatively increasing protein contamination. The recovery of antibody activity in the supernatant was reduced when the OA concentration was increased.

Protein analysis by SDS–PAGE revealed that all supernatants contained mainly $F(ab)_2$ resulting from the digestion of IgG by pepsin. However, the contaminated proteins in the supernatant obtained by precipitation with 1% OA seemed to be more than those with higher concentrations. The precipitate was principally composed of low molecular weight digested proteins other than $F(ab)_2$. When all of the in formation obtained was taken into consideration, an OA concentration of 6% gave the best results, i.e., high antibody activity recovery (70%) and lower protein impurities. It was also observed that at this concentration of OA, a clear supernatant could be obtained by centrifugation at 10,000 × *g* for 10 min. It should be noted that at the concentrations studied, OA did not affect the determination of protein concentration by Lowry's method, neither the assay of antibody activity by bioassay nor the evaluation of protein patterns of SDS–PAGE gels.

3.2. Chromatographic purification of $F(ab)_2$

After acid precipitation the enzyme digested product was loaded to an anion-exchange (Cellufine A-200) column. The feed material

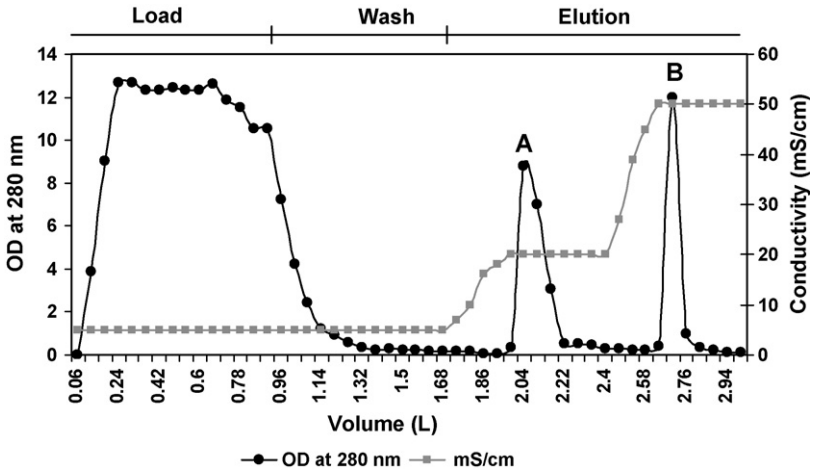

Fig. 1. Chromatographic profile of equine anti-rabies $F(ab)_2$ on Cellufine A-200 ion-exchanger. Antibody fragment did not bind to Cellufine A-200 (0–900 ml) and majority of the contaminant that bound to Cellufine A-200 eluted at 200 mM NaCl (peak A) and 500 mM NaCl (peak B).

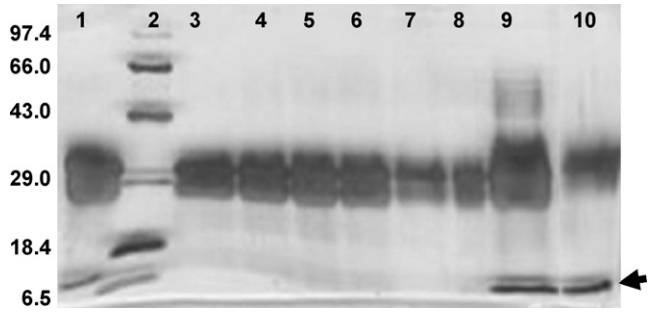

Fig. 2. SDS–PAGE of partially purified $F(ab)_2$ eluted from Cellufine A-200. Lane 1 shows the pre-load to the column, which has the impurity (lower band corresponding to 5.0 kDa). Lanes 3–8 indicate the flow through [$F(ab)_2$] that did not bind to column. Lanes 9 and 10 indicate elutes (contaminating protein bound to media, indicated by arrow). Standard molecular weight marker is loaded in lane 2. Respective molecular weight of each band in kDa unit is indicated.

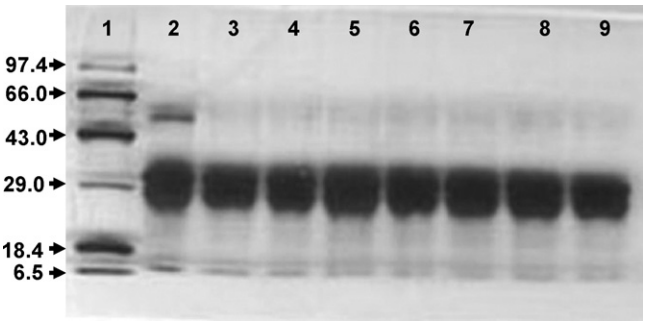

Fig. 4. SDS–PAGE of purified $F(ab)_2$ eluted from ProSep-vA Ultra. Lane 1 shows standard molecular weight marker. Respective molecular weight of each band is indicated in kDa unit. Lane 2 indicates the pre-load to the column, which has the impurity (Fc fragment). Lanes 3–7 indicate the flow through [$F(ab)_2$] that did not bind to column and lane 8 indicates the post-load wash.

contained Fc fragment and other contaminants from serum along with $F(ab)_2$. The $F(ab)_2$ and Fc fragment did not bind to the matrix and came out in flow through. Majority of small molecular impurities bound to the matrix which got eluted as two peaks with increasing salt gradient (Fig. 1). Upon SDS–PAGE analysis it was found that the flow through represented $F(ab)_2$ and Fc fragments (Fig. 2) and the first (A) and second peak (B) represented a majority of other contaminants, which may be digested proteins, i.e., ceruloplasmin and other plasma proteins. Low molecular weight impurity appeared in elution of 200 and 500 mM NaCl. Flow through from Cellufine A-200 resin represented $F(ab)_2$ and Fc band in SDS–PAGE without any low molecular weight impurity. 2.88 g protein impurities bound to 900 ml of cellufine DEAE (A-200). Dynamic binding capacity (DBC) was found to be 7.2 mg ml^{-1}. 84.8% protein recovery observed. The observed DBC is low as the column was not fully saturated, hence can take further more protein load.

The flow through from Cellufine-A column was loaded to ProSep-vA Ultra affinity column. The complete profile of affinity chromatography using ProSep-vA Ultra is presented in Fig. 3. The $F(ab)_2$ fragments did not bind to the resin and came off in the flow through (Fig. 4). SDS–PAGE analysis of the peak eluted off the ProSep-vA Ultra found to have most off the Fc fragments. Based on SDS–PAGE results and mass balance, it is proven that dynamic binding capacity of ProSep-vA Ultra is ~17.8 mg ml^{-1} of media. $F(ab)_2$ of equine origin could be purified using ProSep-vA Ultra in a flow through (negative) chromatography. Purity of $F(ab)_2$ as evidenced from SDS–PAGE is acceptable with more than 85% of desired protein [$F(ab)_2$] recovery. Fractions of purified $F(ab)_2$ was pooled and concentrated to 1500 IU ml^{-1} and 8× diafiltration was done to bring the product to formulation buffer. The mass balance and total product recovery during the chromatographic purifications is presented in Table 1. The biological activity (IU ml^{-1}) of $F(ab)_2$ increased with every step of purification. This offers an indirect correlation of purity.

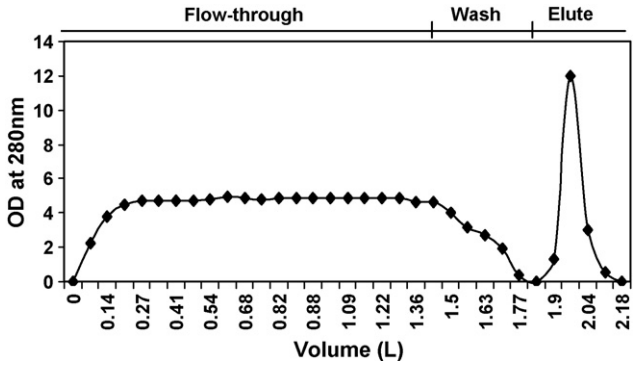

Fig. 3. Chromatogram of affinity chromatography using ProSep-vA Ultra. Antibody fragment [$F(ab)_2$] did not bind to ProSep-vA Ultra (fraction 0–1.4 l) and majority of the contaminants [Fc fragment] that bound to the affinity matrix eluted at pH 3.0 (fraction 1.84–2.18 l).

We obtained low dynamic binding capacity of Fc to ProSep-vA Ultra resin. This could be due to poor affinity of equine IgG subtypes in comparison to human IgG [40]. Moreover, the media was not saturated and break through curve was not established in the present study, leaving possible scope for further enhancement in the dynamic binding capacity of ProSep-vA Ultra. Throughout the process of affinity chromatography using ProSep-vA Ultra, the linear velocity was kept constant at 253 cm h^{-1}. ProSep-vA Ultra is a non-compressible resin with a base matrix of controlled pore glass. ProSep-vA Ultra had been used successfully for purification of IgG at higher linear velocities [41]. Further process compression is possible if the linear velocity during equilibration, washing and elution can be increased to 800 cm h^{-1} or higher. In the present study, we have not evaluated the impact of different residence times during sample loading. From a process scale-up point of view, further studies on different residence times may offer economical process design.

3.3. *Characterization of purified F(ab)$_2$*

Purified $F(ab)_2$ was separated by SDS–PAGE and the purified protein migrated with an apparent molecular mass of ~100,000 to 110,000 Da under non-reducing conditions where as under reducing conditions, they are present as hands of 25,000–31,000 Da. Densitometric scanning of the silver stained SDS–PAGE gels indicated that the purity of refolded protein is greater than 98%. Purified $F(ab)_2$ was characterized using a variety of biochemical and biophysical methods. The homogeneity of purified $F(ab)_2$ was analyzed by size exclusion chromatography (SEC-HPLC). Purified $F(ab)_2$ elutes in a single, symmetric peak by size exclusion chromatography on a TSK Gel-3000 SWXL HPLC-SEC column suggesting that purified $F(ab)_2$ is homogenous (Fig. 5). The retention time of purified equine antirabies $F(ab)_2$ on an TSK Gel-3000 SWXL column is consistent with an apparent molecular mass of 50 kDa indicating that purified $F(ab)_2$ does not contain aggregates or multimers. Comparative HPLC analysis and SDS–PAGE of our chromatographically purified $F(ab)_2$ with commercially available purified $F(ab)_2$ manufactured by European and Indian manufacturer for therapeutic use indicate that the present reported product has best purity in comparison to the other tested commercial product (Fig. 6A and B). Potency of the purified $F(ab)_2$ was measured by using Bio-Rad ELISA

Table 1
Mass balance and purification index of $F(ab)_2$ purified from hyperimmune equine sera.

Fraction	Volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Activity (IU ml^{-1})	Total activity (IU)	Specific activity (IU/mg)	Yield (% activity recovered)
Plasma	350.00	80.00	25600.00	1000.00	350000.00	13.67	100.00
After pepsin digestion	718.00	34.90	25058.00	428.00	307304.00	12.26	87.80
Supernatant after octanoic precipitation	700.00	25.57	17889.00	350.00	245000.00	13.70	70.00
Concentration by ultrafiltration	1075.00	15.00	16125.00	221.00	237650.00	14.74	67.90
Cellufine A-200 pre-load	900.00	15.00	13500.00	221.00	198900.00	14.73	56.83
Cellufine A-200 flowthrough + wash	1700.00	6.13	10530.00	99.21	168667.20	16.02	48.19
ProSep-vA Ultra pre-load	1400.00	7.00	9800.00	116.86	163607.00	16.69	46.74
ProSep-vA Ultra flowthrough + wash	1840.00	4.15	7644.00	81.80	150518.44	19.69	43.01
Concentration by ultrafiltration	95.00	14.32	1352.00	1500.00	142500.00	105.40	40.71

Total protein = protein (mg) × total volume (ml). Total activity = activity (IU) × total volume (ml). Specific activity = total activity. (IU)/total protein (mg). Yield = [total activity of the sample/initial total activity] × 100. All values are mean of three independent trials/studies.

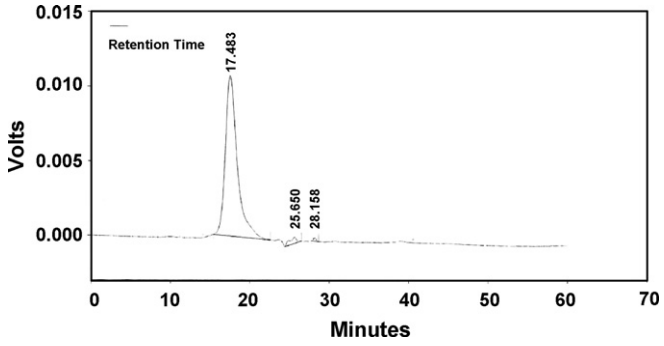

Fig. 5. HPLC analysis of purified $F(ab)_2$. Elution profile of $F(ab)_2$ as single homogenous peak represents >99% purity of the product. Column: SEC, TSK Gel-3000 SWXL (TosoHass). Sample: 20 μl of purified $F(ab)_2$; eluent: 20 mM phosphate buffer in 150 mM sodium chloride solution, pH 7.2, Detection at 280 nm; flow-rate: 1 ml min^{-1}.

Kit. It was found to be 1500 IU/ml. Purity and reactivity of purified $F(ab)_2$ was also identified by immunoprecipitation. ProSep-vA Ultra purified $F(ab)_2$ resulted in a single arc in immunoprecipitation (Fig. 7) indicating purity and reactivity. Based on the immunoprecipitation study, we found that our purified $F(ab)_2$ was better than the commercially available purified $F(ab)_2$. However, immunopre-

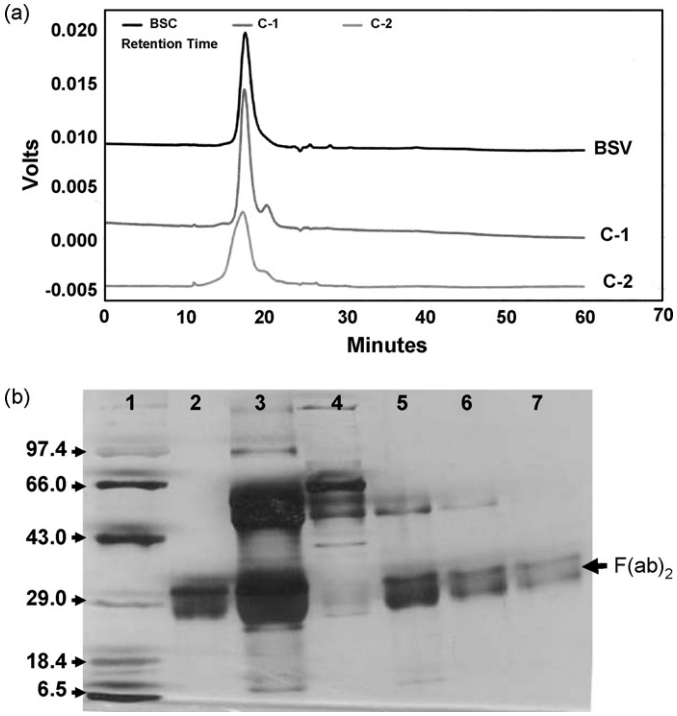

Fig. 6. (A) Comparative analysis of purity of $F(ab)_2$. Elution profile of purified $F(ab)_2$ along with two different commercially available $F(ab)_2$ produced by Indian manufacturers, code name C-1 and C-2 were superimposed on a single frame. Comparatively, the presently purified $F(ab)_2$ has better purity (BSV) than commercially available $F(ab)_2$ (C-1, C-2). Column: SEC, TSK Gel-3000 SWXL (TosoHass). Sample: 20 μl of purified $F(ab)_2$; eluent: 20 mM phosphate buffer in 150 mM sodium chloride solution, pH 7.2, Detection at 280 nm; flow-rate: 1 ml min^{-1}. (B) SDS–PAGE showing purity of $F(ab)_2$ after each step of purification and comparative analysis of purity of $F(ab)_2$ against commercially available $F(ab)_2$, Lane 1 shows standard molecular weight marker is loaded in lane 1. Respective molecular weight of each band is indicated in kDa unit. Lane 2 and 3 indicate commercially available anti-rabies $F(ab)_2$ produced by European and Indian manufacturers respectively. Lane 4 represents equine hyperimmune sera, Lane 5–7 indicate improvement in purity of F(ab)2 after octanoic acid precipitation (lane 5), after ion-exchange chromatography (lane 6) and after affinity chromatography using ProSep-vA Ultra (lane 7).

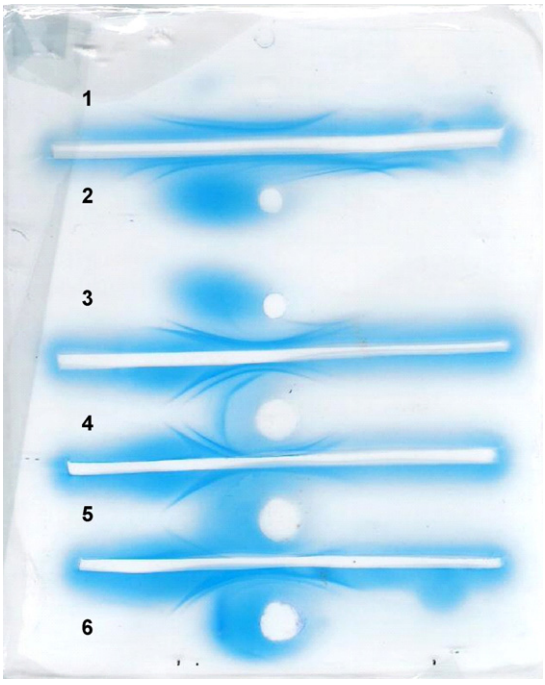

Fig. 7. Comparative analysis of purity and reactivity of $F(ab)_2$ by immunoprecipitation. Well 1 shows single arc of Pasteur $F(ab)_2$ standard (positive control). Wells 2 and 3 contain commercially manufactured equine antiserum and precipitated antisera respectively showing numbers of precipitated arc indicating impure sample. Well 4 contains Cellufine A-200 post-load sample showing two arcs indicating partially purified $F(ab)_2$. Well 5 contains ProSep-vA Ultra purified post-load sample showing single arc indicating highly purified $F(ab)_2$ without any impurities and desired reactivity. Well 6 contains ProSep-vA Ultra elute which shows again multiple arcs indicating impurities which is bound to ProSep-vA Ultra media.

cipitation is a qualitative study and does not offer insight on purity or contaminant profile of the product. We have not performed exact quantization of contaminants. From safety and quality point of view further studies on contaminant profiling of finished product may offer additional assurance of finished product quality.

4. Conclusion

In conclusion, we have purified biologically functional $F(ab)_2$ of equine origin. We have established that F(ab)2 could be purified by the combination of anion-exchange and protein A-based affinity chromatography. Though we have demonstrated the proof of concept, a large-scale process development study is required to demonstrate scalability and industrial-scale feasibility. Chromatography-based commercial-scale purification of equine immunoglobulin has so far not been practiced due to high cost of operation. Since the process discussed uses controlled pore glass based non-compressible chromatographic matrix, with due process optimization, inexpensive scale-up can be established. Moreover, ProSep-vA Ultra is known for longer life of resin [42], incompressible bed height, allowing high flow-rate and diagonal scale-up (higher bed height). These features may offer a suitable operational window for economical production of high quality equine immunoglobulin (IgG) or immunoglobulin fragments [$F(ab)_2$]. Further we demonstrated that the purified $F(ab)_2$ has the desired purity, specificity and potency. This method could be valuable for the development of other $F(ab)_2$ of different origin (sheep, goat, etc.) or $F(ab)_2$ developed against different targets (anti-tetanus, anti-snake venom, anti-scorpion venom, etc.) for commercial production.

Acknowledgements

We thank Mr. Kakashaeb Lonkar and Mr. Krishna Khapare for their generous help and support in analysis. Help rendered by Mr. Premjeet Rajan and Mr. Prafulchand Achar is acknowledged. We thank Mr. M.S. Mahadevan, Vice President, Millipore India and board of management of Bharat Serum and Vaccines Ltd., for offering all facilities and support required for this study.

References

[1] T. Burnouf, E. Griffiths, A. Padilla, S. Seddik, A.S. Marco, J. Gutierrez, Biologicals 32 (2004) 115.
[2] H. Wilde, T. Penamas, V. Sitprija, N. Chaiyabutr, Ann. Intern. Med. 125 (1996) 233.
[3] M.C. dos Santos, L.M.R. D'Império, G.C. Furtado, G.M. Colletto, T.L. Kipnis, W. Dias da Silva, Toxicon 27 (1989) 297.
[4] C. Newcombe, A.R. Newcombe, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 848 (2007) 2.
[5] D.G. Lalloo, R.D.G. Theakston, J. Toxicol. Clin. Toxicol. 41 (2003) 277.
[6] R.D.G. Theakston, D.A. Warrell, Toxicon 29 (1991) 1419.
[7] R.D.G. Theakston, D.A. Warrell, E. Griffiths, Toxicon 41 (2003) 541.
[8] J. Goudsmit, W.E. Marissen, W.C. Weldon, M. Niezgoda, C.A. Hanlon, A.B. Rice, J. de Kruif, B. Dietzschold, A.B.H. Bakker, C.E. Rupprecht, J. Infect. Dis. 193 (2006) 796.
[9] J. Lang, L. Kamga-Fotso, J.C. Peyrieux, C. Blondeau, C. Lutsch, R. Forrat, Am. J. Trop. Med. Hyg. 63 (2000) 298.
[10] M. García, M. Monge, G. León, S. Lizano, E. Segura, G. Solano, G. Rojas, J.M. Gutiérrez, Biologicals 30 (2002) 143.
[11] H. Wilde, S. Chutivongse, Am. J. Trop. Med. Hyg. 42 (1990) 175.
[12] H. Wilde, P. Thipkong, V. Sitprija, N.C. Butr, Ann. Intern. Med. 125 (1996) 233.
[13] L. Wang, X. Sun, R. Ghosh, Biotechnol. Bioeng. 99 (2007) 625.
[14] R.D.G. Theakston, D.A. Warrell, Lancet 356 (2000) 2104.
[15] C.G. Pope, Br. J. Exp. Pathol. 20 (1939) 32.
[16] G. Rojas, J.M. Jimenez, J.M. Gutiérrez, Toxicon 32 (1993) 351.
[17] R. Raweerith, K. Ratanabanangkoon, J. Immunol. Methods 282 (2003) 63.
[18] P. Kumpalume, N.K.H. Slater, J. Chromatogr. A 1022 (2004) 41.
[19] I. Fernandes, E.X. Lima, H.A. Takehara, A.M. Mouradasilva, I. Tanjoni, J.M. Gutiérrez, Toxicon 38 (2000) 633.
[20] R. Ghosh, J. Membr. Sci. 237 (2004) 109.
[21] L. Wang, D.M. Kanani, R. Ghosh, J. Immunol. Methods 314 (2006) 1.
[22] D.G. Covell, J. Barbet, O.D. Holton, C.D. Black, R.J. Parker, J.N. Weinstein, Cancer Res. 46 (1986) 3969.
[23] J.M. Scherrmann, J. Toxicol. Clin. Toxicol. 32 (1994) 363.
[24] C.G. Pope, Br. J. Exp. Pathol. 20 (1939) 132.
[25] A.J. Harms, Biochem. J. 42 (1948) 390.
[26] M. Steinbuch, R. Audran, Arch. Biochem. Biophys. 134 (1969) 279.
[27] A.C. Roque, C.R. Lowe, Biotechnol. Adv. 24 (2006) 17.
[28] A. Willems, J. Leoen, S. Schoonooghe, J. Grooten, N. Mertens, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 786 (2003) 161.
[29] H. Wlad, A. Ballagi, L. Bouakaz, Z. Gu, J.C. Janson, Protein Expr. Purif. 22 (2001) 325.
[30] P.A. Christensen, in: Chen-yuan Lee (Ed.), Handbook of Experimental Pharmacology, vol. 52, 1979, p. 825 (Chapter 20).
[31] C. Chotwiwatthanakun, R. Pratanaphan, S. Akesowan, S. Sriprapat, K. Ratanabanangkoon, Toxicon 39 (2001) 1487.
[32] F. Cliquet, L.M. McElhinney, A. Servat, J.M. Boucher, J.P. Lowings, T. Goddard, K.L. Mansfield, A.R. Fooks, J. Virol. Methods 117 (2004) 1.
[33] A. Servat, F. Cliquet, Virus Res. 120 (2006) 17.
[34] A. Servat, M. Feyssaguet, I. Blanchard, J.L. Morize, J.L. Schereffer, F. Boue, F. Cliquet, J. Immunol. Methods 318 (2007) 1.
[35] A. Servat, A. Labadie, A. Hamen, F. Boue, F. Cliquet, Biologicals 36 (2008) 19.
[36] G. León, M. Monge, E. Rojas, B. Lomonte, J.M. Gutiérrez, Toxicon 39 (2001) 793.
[37] A. Padilla, T. Govezensky, L.D. Possani, C. Larralde, Toxicon 46 (2005) 142.
[38] R.G. Jones, T.A. Alsop, R. Hull, R. Tierney, P. Rigsby, J. Holley, D. Sesardic, Toxicon 48 (2006) 246.
[39] A. Ryle, in: G. Perlmann, L. Lerand (Eds.), Methods in Enzymology, vol. 19, Academic Press, NY, 1970, p. 316.
[40] M.J. Lewis, B. Wagner, J.M. Woof, Mol. Immunol. 45 (2008) 818.
[41] J.T. McCue, G. Kemp, D. Low, I. Quinones-Garcia, J. Chromatogr. A 989 (2003) 139.
[42] R.M. O' Learly, D. Feuerhelm, D. Peers, Y. Xu, G.S. Blank, Biopharm. Int. 14 (2001) 10.