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Research paper

New method to produce equine antirabies immunoglobulin F(ab′)$_2$ fragments from crude plasma in high quality and yield

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ABSTRACT

Rabies is still a major cause of human deaths in several developing countries. According to the World Health Organization, administration of antirabies serum or antirabies immunoglobulin is recommended for patients who have experienced a category-III exposure to rabies. Improvement of antirabies immunoglobulin production is required to enhance safety and efficacy of the products. In this paper, a new method to produce equine antirabies immunoglobulin F(ab′)$_2$ fragments from crude plasma is proposed. First, protein G affinity chromatography was used to purify IgG from equine plasma. Moreover, purification of IgG was shown to facilitate its digestion by pepsin. Compared to the direct digestion of crude plasma, a lower amount of pepsin and a shorter digestion time were required to completely digest the purified IgG to F(ab′)$_2$. Complete digestion of purified IgG to F(ab′)$_2$ was achieved at a pepsin/IgG (w/w) ratio of 5:45 with preservation of structure and potency. Finally, purification of F(ab′)$_2$ was accomplished by a combination of protein A affinity chromatography and ultrafiltration with a 50-kDa nominal molecular weight cut-off membrane. The new process resulted in 68.9 ± 0.6 (%) total recovery of F(ab′)$_2$ and a F(ab′)$_2$ product of high potency.

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1. Introduction

Passive immunization of patients against rabies is recommended in areas with high infection rates of the category 3 exposure according to the World Health Organization [1]. There are different types of products available for passive immunization: human and animal (mainly equine) polyclonal immunoglobulin and antibody fragments thereof. Due to the high costs and limited access to human immunoglobulin, purified horse immunoglobulin products are mainly used in developing countries.

Administration of antirabies F(ab′)$_2$ products is safer and better tolerable for the patients than the complete antirabies IgG molecule, because F(ab′)$_2$ lacks the Fc fragment which can activate complement [2]. Recent studies have shown that not only non-digested IgG but also IgG dimers/aggregates may cause anaphylactic reactions [3,4]. However, one great disadvantage in the production of F(ab′)$_2$ is the need for an enzymatic digestion step, which often results in activity loss of the resulting F(ab′)$_2$ fragment compared to the non-digested IgG. This activity loss can be explained by (i) the aggressive low pH conditions necessary for digestion and (ii) the unspecific digestion of IgG that becomes evident with incubation at longer reaction times [2]. Numerous digestion conditions varying in type and concentration of enzyme and digestion time have been described, and the process is not well standardized. Several enzymes including pepsin, trypsin, and papain have been used for antibody digestion. Among these, pepsin is the only one capable of completely cleaving IgG to give F(ab′)$_2$ [3,5,6]. Therefore, the majority of antirabies products consists of F(ab′)$_2$ antibody fragments prepared by pepsin digestion of hyperimmune equine plasma and subsequent purification.

After the enzymatic digestion, several purification steps are usually required to ensure a high purity and efficacy of the products with minimum side effects. Purification can be performed by salt precipitation using caprylic acid, ammonium sulfate or sodium sulfate, thermocoagulation, diafiltration, ion-exchange chromatography, affinity chromatography, and/or a combination of these methodologies [6–8]. Generally, salt precipitation is inexpensive, but it results in a low antibody recovery [7,9,10]. The process is

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difficult to scale up under sterile conditions and gives low yield and purity [11–13]. Additionally, it may be associated with large losses in antibody activity and/or aggregate formation [6]. Alternatively, caprylic acid precipitation can increase the yield of IgG, but the process is time-consuming and does not eradicate endotoxin-producing bacterial contaminations [9]. Though effective, diafiltration fails to completely remove pepsin, which can affect the stability of the antibody fragments [5].

The current method for the preparation of $F(ab')_2$ antirabies serum approved by WHO is based on the approach described by Pope [14,15] and Harms [7]. Briefly, pepsin digestion (30 min, 30 °C, pH 3.2) of hyperimmune crude equine plasma is followed by a heat denaturation step (1 h at 55 °C, pH 4.3) to precipitate most of non-IgG and by a further addition of ammonium sulfate to precipitate the IgGs including $F(ab')_2$. Because of the salt precipitation step, the process results in $F(ab')_2$ products of comparably low yield and low purity.

The objective of our research was to develop a new, scalable process for the digestion and purification of antirabies $F(ab')_2$ fragment from equine crude plasma with the goal to obtain a higher yield than with the WHO process, as well as a highly active and pure product. Process development was accompanied by aggregate analysis, structural characterization, and activity testing of the $F(ab')_2$ fragments.

2. Materials and methods

2.1. Materials

Horse crude plasma and equine antirabies immunoglobulin (TRCS ERIG) product (lot RF 01509) were obtained from Queen Saovabha Memorial Institute, the Thai Red Cross Society (Bangkok, Thailand). Pepsin from dog stomach was reagent grade (specific activity 654 units/mg protein) and purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Also NaCl, Na_2HPO_4, $NaH_2PO_4{\cdot}2H_2O$, and tris(hydroxymethyl)aminomethane were from Sigma–Aldrich (Zwijndrecht, The Netherlands). Glycine and sodium sulfate were from Merck (Darmstadt, Germany). Sodium azide was from Fluka (Zwijndrecht, The Netherlands).

2.2. Purification of IgG from crude plasma by protein G affinity chromatography

Protein G is a cell surface-associated protein from streptococcus that binds to most mammalian immunoglobulins primarily through their Fc regions and light chain [16]. Protein G NAB™ Spin columns (Thermoscientific, Etten-Leur, The Netherlands) containing 1-ml resin bed of protein G agarose were used. Buffers were filtered through 0.2-μm Millex® filters (Millipore, Ireland). The purification experiments were carried out at room temperature. To purify IgG from crude plasma, the crude plasma was first diluted at a 1:1 (v/v) ratio with dilution buffer (0.1 M phosphate, 0.15 M sodium chloride, pH 7.2). Two milliliters of the diluted plasma was then loaded onto a protein G column pre-equilibrated with 2 ml of binding buffer (repeated three times), and the column gently turned with end-over-end turning for 15 min. Unbound plasma components were washed from the column with the binding buffer until the UV absorbance of the eluted solution at 280 nm reached zero. The fractions from the washing step were discarded. The bound IgG was eluted from the column using eight times 1 ml elution buffer (0.1 M glycine, pH 3.0, 3.5 and 4.0). The eluted fractions were pooled, the absorbance at UV 280 nm was determined, and the IgG concentration was calculated based on an extinction coefficient of 1.5 ml mg^{-1} cm^{-1} for IgG [17].

2.3. Peptic digestion of crude plasma and purified IgG

Peptic digestion of crude plasma was carried out at protein concentration of 20 mg/ml and pepsin-to-protein ratios from 1:45 to 90:45 (w/w) for 30 min. In addition, the digestion time of pepsin-to-protein ratios at 30:45 was varied from 0.5 to 24 h. Before digestion, the pH of the crude plasma solution was adjusted to 3.2 by 0.4 N HCl. The digestion procedure was carried out using 10 ml solution in a 50-ml tube at 37 °C in a shaking water bath under continuous agitation at 130 rpm. The digestion was stopped by adding 0.5 ml of 0.4 N NaOH to increase the pH to 7.

Similarly, the peptic digestion of the purified IgG was performed as described above at IgG concentration of 1 mg/ml and pepsin-to-IgG ratios of 1:45, 3:45, and 5:45 (w/w) at 37 °C for 30 min.

2.4. Purification of $F(ab')_2$ by protein A affinity chromatography and ultrafiltration

After the pepsin digestion step, undigested IgG, pepsin, and low molecular weight digestion products were removed by protein A purification and ultrafiltration. Protein A is a cell surface-associated protein from the bacterium *Staphylococcus aureus* that binds to Fc regions of antibodies which has led to its widespread use as a powerful affinity ligand for several immunological and purification applications [18,19]. Protein A was used instead of protein G, because $F(ab')_2$ was found to interact with protein G (results not shown). One milliliter of digested sample was loaded onto a protein A NAB™ Spin column (Thermoscientific, Etten-Leur, The Netherlands), and the purification process was carried out using the above-described protein G purification protocol. Contrary to the protein G purification protocol, the fractions of non-bound protein and the washing solution were collected, as those contain the $F(ab')_2$. Those fractions were pooled and the concentration determined from the UV absorbance at 280 nm using an extinction coefficient of 1.45 ml mg^{-1} cm^{-1} for $F(ab')_2$ [20]. Elution fractions containing undigested IgG were discarded.

Prior to ultrafiltration carried out by the ultracentrifugation membrane Amicon Ultra-4 (Millipore Corporation, MA) with a 4-ml centrifuge concentrator with a 50-kDa nominal molecular weight cutoff (MWCO), the cellulose membrane was pre-rinsed with 0.1 N NaOH followed by a second washing with phosphate buffer pH 7.2. Ten milliliters of the samples was loaded onto the ultrafiltration membrane and centrifuged at 3270*g* for 3 min using the device, which reduced the volume of the sample to 4 ml. Then, 5 ml of phosphate buffer pH 7.2 was added to the ultrafiltration device, and it was centrifuged again. This procedure was repeated two times.

2.5. UV spectroscopy

An Agilent 8453 UV–Vis spectrometer (Agilent, Waldbronn, Germany) was used for UV measurements. The measurements were performed using 1 ml of each solution in a 1-cm path length quartz cuvette. The spectra of the samples were corrected for the absorbance of the corresponding placebo solutions.

2.6. Sodium dodecyl sulfate–poly acrylamide gel electrophoresis (SDS–PAGE)

Non-reducing SDS–PAGE was used to separate proteins according to their molecular weight [21] using a 7.5% Tris–HCl gel (Bio-Rad Laboratories, Veenendaal, The Netherlands). The samples (10 μl per lane) were applied and subjected to electrophoresis at 140 V (volt) for approximately 50 min. A mixture of broad-ranged maker proteins of known molecular weights

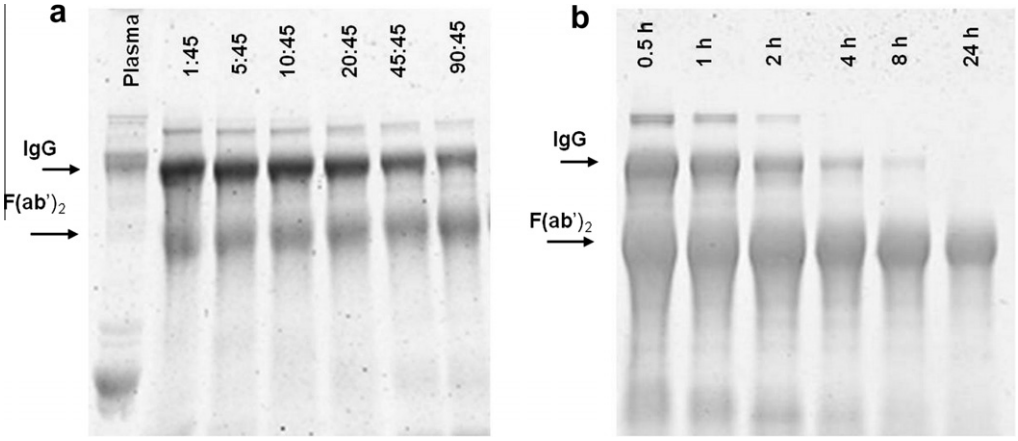

Fig. 1. Non-reducing SDS-PAGE showing the effect on digestion of IgG to F(ab′)$_2$ at pH 3.2 of (a) pepsin-to-protein ratio (w/w) at a digestion time of 30 min and (b) digestion time at a pepsin-to-protein of 30:45 (w/w).

(Bio-Rad Laboratories, Veenendaal, The Netherlands) was also run with the protein samples. Protein bands were visualized using Coomassie blue staining.

2.7. Western blotting

After SDS–PAGE analysis, the proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (BA85, 0.45 μm, Schleicher Schuell, Dassel, Germany) in a Trans-Blot cell (Bio-Rad Chemical Division, South Richmond, CA) at 100 V for 2 h, at 4 °C. Utilizing the protocol of Hermeling et al. [22], 0.005% (w/v) of polysorbate 20 in PBS (3.6 mM KH_2PO_4, 6.4 mM Na_2HPO_4 and 145 mM NaCl at pH 7.2) was used as washing buffer and 8% (w/v) non-fat milk powder in PBS/polysorbate 20 as blocking buffer. After the transfer step, the nitrocellulose sheet was incubated in blocking solution and, subsequently, washed using washing buffer. The blocked membrane was incubated overnight at 2–8 °C with horse radish peroxidase (HRP)-labeled goat anti-horse polyclonal antibody (LS-CC59914/18439, Lifespan Biosciences, Seattle, WA) to allow binding of the secondary antibody to the intact F(ab′)$_2$ fragments. For color development, the washed sheet was soaked in a freshly prepared substrate solution, consisting of 4-chloro-1-naphthol (Sigma–Aldrich, St. Louis, MO) in methanol and 5 μl of 30% hydrogen peroxide. The reaction was terminated by rinsing the stained membrane with water.

2.8. High-performance size exclusion chromatography (HP-SEC)

Monomer, fragments and soluble aggregates of IgG and F(ab′)$_2$ were determined by HP-SEC (Waters 717 plus Autosampler, Milford, MA) and UV detection at 280 nm (model SPD-6AV, Shimadzu Corporation, Kyoto, Japan). A TSK Gel 3000_{SWXL} column (300 × 7.8 mm, Tosoh Biosep, Stuttgart, Germany) with a TSK Gel 3000 precolumn (Tosoh Biosep, Stuttgart, Germany) was used. One hundred microliters of the samples was injected, and the separation was performed at a flow rate of 0.3 ml/min. The running buffer was composed of 100 mM phosphate, 100 mM sodium sulfate, and 0.025% (w/v) sodium azide at pH 7.2.

2.9. Far-UV circular dichroism (far-UV CD) spectroscopy

Far-UV CD spectra were measured with a Jasco CD spectropolarimeter (model J-715, Jasco, Tokyo, Japan) in a 2-mm path length quartz cuvette at 25 °C. Prior to the measurement, each sample was diluted to a concentration of 0.2 mg/ml F(ab′)$_2$. The CD spectra were collected from 190 to 250 nm at a speed of 20 nm/min, steps of 0.2 nm, a response time of 2.0 s, and a bandwidth of 2 nm (average of five scans). The spectra were background corrected for the spectrum of the solvent, and data were calculated as mean residual ellipticity ($[\theta]_{mrw}$) base on a mean amino acid residue weight of 110 assumed for F(ab′)$_2$ [23]. The mean residue ellipticity was determined according to as $[\theta]_{mrw,\lambda} = (MRW \times \theta_\lambda)/(10 \times c \times d)$, where MRW is the mean residual weight, θ_λ is the observed ellipticity in millidegrees at wavelength (λ), c is the protein concentration in mg/ml, and d is the path length in cm [24].

2.10. Fluorescence spectroscopy

Intrinsic fluorescence emission spectra were recorded on a FS920 fluorescence spectrometer (Edinburg Instruments, UK) at 25 °C using a 1-cm quartz cuvette. Each sample was diluted to a concentration of 0.1 mg/ml. Excitation was performed at 295 nm to selectively excite Trp, and emission spectra were collected from 310 to 450 nm. The slits for excitation and emission were 2.5 nm, while the scan speed was 100 nm/min. Fluorescence spectra were corrected for the background spectrum of solvent.

2.11. Potency assay

Potency of antirabies serum was determined by the rapid fluorescent focus inhibition test (RFFIT) [25]. Each serum sample (crude plasma, purified IgG, digested samples or purified F(ab′)$_2$ was diluted twofold with maintenance medium that contained 2% FBS (fetal bovine serum) and placed in a 96-well microplate. The rabies virus was added to each well and incubated in a 5% CO_2 incubator at 37 °C for 90 min. Next, BHK-21 cells were added to each well and incubated for 24 h. Finally, cells were fixed with 80% v/v acetone and stained with a fluorescent antibody in order to detect the presence of non-neutralized virus (fluorescent foci) under a fluorescence microscope. The RFFIT test was used to determine a value for the rabies virus neutralizing antibody concentration (IU/ml). The IU stands for international unit and was calculated from the titer by comparison with WHO international standard of antirabies immunoglobulin. In addition, the potency recovery of each sample was expressed as a percentage of the relative activity of the sample against the activity of the crude plasma ($n = 3$).

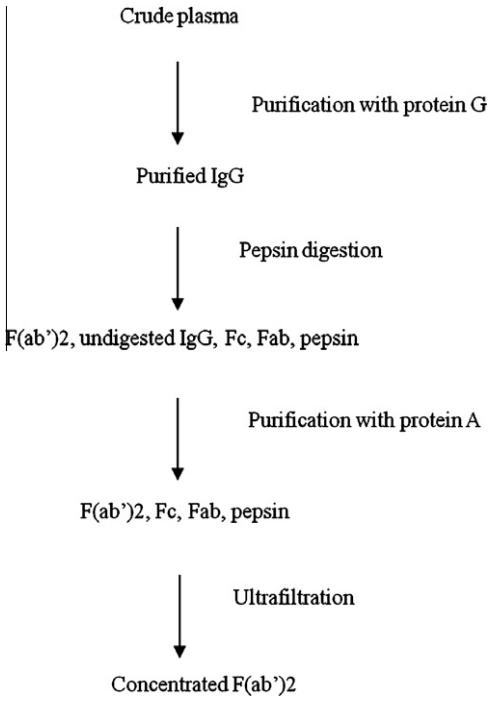

Fig. 2. Flow sheet of the production process developed within our study.

3. Results and discussion

3.1. Direct digestion of horse crude plasma

The effect of pepsin amount and reaction time on the digestion of horse crude plasma at pH 3.2 was studied in different sets of experiments. IgG present in crude plasma could not be completely digested within 30-min digestion time using pepsin-to-protein ratios varying from 1:45 to 90:45 (w/w) (Fig. 1a). Moreover, for a fixed ratio of pepsin to protein 30:45 (w/w), IgG was still present after 8-h reaction time (Fig. 1b). Overall, high amounts of pepsin and long digestion times were required for complete IgG digestion from crude plasma, illustrating that this approach is highly inefficient.

3.2. Optimization of $F(ab')_2$ purification and digestion

In order to optimize the production of $F(ab')_2$, we decided to purify IgG from the crude plasma prior to the digestion process. Fig. 2 gives an overview of the different steps in the new $F(ab')_2$ production process, which we used in our study for the purification and digestion of horse crude plasma.

3.3. Purification of IgG from horse plasma by protein G affinity chromatography

Protein G affinity chromatography was used to purify IgG from crude plasma. The effect of pH of elution buffer (pH 3.0, 3.5 and 4.0) on the monomer content of purified IgG after using protein G affinity chromatography was tested. After elution at pH 3.0, HP-SEC showed that only 89.6% monomer (7.8% dimers, 2.6% larger aggregates) was recovered, compared to 95.3% monomer (3.6% dimers, 1.9% larger aggregates) after elution at pH 3.5. Elution at pH 4.0 was not feasible, as it resulted in very low recoveries (data not shown). Based on these results, the elution buffer at pH 3.5 was chosen to elute horse IgG from protein G column.

SDS–PAGE showed that protein G affinity chromatography was effective in purifying horse IgG from crude plasma (Fig. 3a, lane 1 and 2) as non-IgG crude plasma proteins could not be detected after purification. The SDS gel did not indicate the existence of covalent IgG aggregates, suggesting that the aggregates observed by HP-SEC were non-covalent aggregates. These aggregates could not be removed by filtration (0.2 μm) or centrifugation (3000*g*, 15 min) since the dimers and larger aggregates were still observed in HP-SEC (data not shown). Moreover, the non-covalent aggregates were still present after adjusting the pH of the purified IgG from pH 3.5 to neutral pH (6.5 or 7.0) by using 1 M Tris buffer (pH 8.5) (data not shown). As the next step was the digestion of the purified IgG by pepsin at pH 3.2, no pH neutralization step was included, but the digestion process was directly continued.

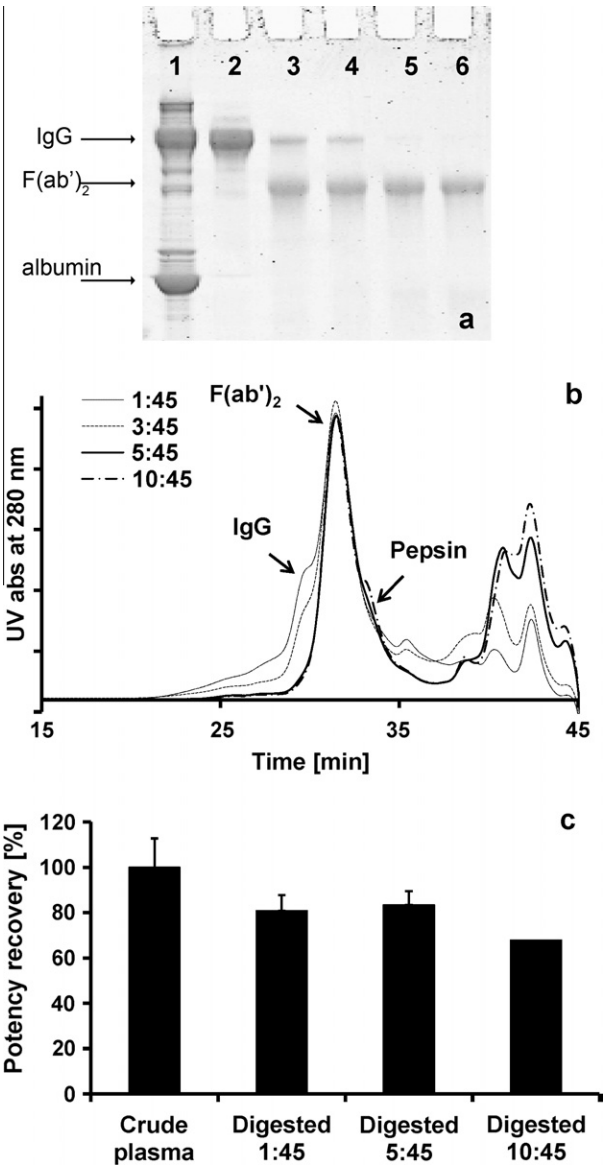

Fig. 3. (a) SDS-PAGE of crude plasma (lane 1), purified IgG (lane 2), digested formulations at pH 3.2 within 30 min at different pepsin-to-protein ratios (w/w) (lanes 3–6). Lane 3: 1:45, lane 4: 3:45, lane 5: 5:45, lane 6: 10:45. (b) HP-SEC chromatograms of the digested samples, at flow rate of 0.3 ml/min. The peaks were assigned based on analysis of purified IgG and pepsin (results not shown). (c) Potency recovery (%) of the digested samples using RFFIT test.

3.4. Peptic digestion of purified IgG

The effect of the pepsin-to-IgG ratio on the digestion process at 37 °C for 30-min reaction time was studied by varying the

pepsin-to-IgG ratio from 1:45 to 10:45 (w/w). A pepsin/IgG (w/w) ratio of 5:45 could completely digest the purified IgG to $F(ab')_2$ in 30-min reaction time (Fig. 3a). Compared to the pepsin digestion of crude plasma (Fig. 1a), a much lower amount of pepsin was required to complete IgG digestion within 30 min. So, to improve the digestion efficiency, it is beneficial to purify IgG from crude plasma before digestion.

From HP-SEC of the digested samples (Fig. 3b), a lower amount of IgG (elution time 29 min) was observed when increasing pepsin amounts were used. However, the $F(ab')_2$ peak (elution time 31 min) was not increasing accordingly. Moreover, pepsin (elution time at 33 min) can be detected at the highest pepsin-to-protein ratio of 10:45 (w/w). After digestion, the non-covalent aggregates present in the purified IgG after protein G affinity chromatography had disappeared. Therefore, it can be concluded that a removal of the aggregates of the IgG after protein G purification is not necessarily required. Furthermore, the RFFIT results (Fig. 3c) show a lower potency recovery (%) with increasing pepsin amount. A potency recovery of about 81% and 84% was found for the pepsin-to-protein ratios of 1:45 and 5:45 (w/w), respectively, whereas the potency recovery was reduced to about 68% for the 10:45 (w/w) condition. It can be concluded that a pepsin-to-protein ratio of 5:45 (w/w) is most suitable for a complete digestion of the purified IgG within 30 min and a good potency recovery of the $F(ab')_2$ fragments.

3.5. Purification $F(ab')_2$

Protein A affinity chromatography can be used to separate undigested IgG and Fc after peptic digestion from $F(ab')_2$. Horse IgG binds to protein A via the Fc region of immunoglobulin molecules, allowing the undigested IgG and Fc to be captured on the column, whereas $F(ab')_2$ is eluting in the non-bound fraction [18,19].

In a previous study, 1.5 M glycine + 3 M NaCl, adjusted to pH 8.9, was used as binding buffer for the purification of a monoclonal antibody with protein A affinity chromatography [10]. Within our study, 1.5 M glycine + 3 M NaCl pH 8.9 and PBS pH 7.2 were compared as binding buffers for the purification of horse $F(ab')_2$ from undigested IgG on the protein A column. From SDS–PAGE (data not shown), it was obvious that 1.5 M glycine + 3 M NaCl can promote the complete binding of IgG to the protein A at the loading concentration of 1 mg/ml, whereas a reduced binding capacity for IgG was found when using PBS pH 7.2. Moreover, at loading concentration of 0.5 mg/ml, complete binding of IgG to protein A was observed when using both buffers. However, using 1.5 M glycine + 3 M NaCl appeared to induce the higher formation of $F(ab')_2$ aggregates when compared to using PBS pH 7.2 as obvious from HP-SEC (Fig. 4). Therefore, PBS pH 7.2 was chosen as binding buffer for the protein A affinity chromatography.

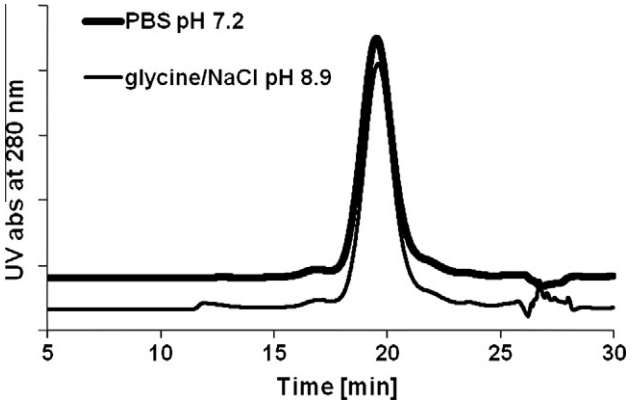

Fig. 4. HP-SEC chromatograms with UV detection at 280 nm of the IgG in different elution conditions at flow rate of 0.5 ml/min.

3.6. Evaluation of $F(ab')_2$ recovery and potency at the different process steps

After optimizing the different purification and digestion process steps, we decided (1) to elute IgG from the protein G affinity chromatography at pH 3.5, (2) to use a ratio of pepsin to protein of 5:45 (w/w) to digest IgG within 30 min at 37 °C, (3) to employ PBS pH 7.2 as binding buffer for protein A affinity chromatography, and (4) to filtrate and concentrate the purified $F(ab')_2$ with centrifugal ultrafiltration using a cellulose membrane (MWCO 50 kDa). Three independent batches were prepared using the novel process conditions.

Table 1 shows the cumulative molar recovery (%) after each purification step of the three batches. The average molar recoveries of $F(ab')_2$ after the full purification process were very reproducible

Table 1
The cumulative molar recovery (%) of fractions obtained from the successive process steps.

Sample	Lot No.	Molar Recovery (%)
Crude plasma	—	100
Purified IgG (protein G)	Lot 1	80.0
	Lot 2	81.5
	Lot 3	81.7
Digested sample (pepsin digestion)	Lot 1	82.9
	Lot 2	84.6
	Lot 3	84.1
Purified $F(ab')_2$ (protein A, ultrafiltration)	Lot 1	69.5
	Lot 2	68.4
	Lot 3	68.7

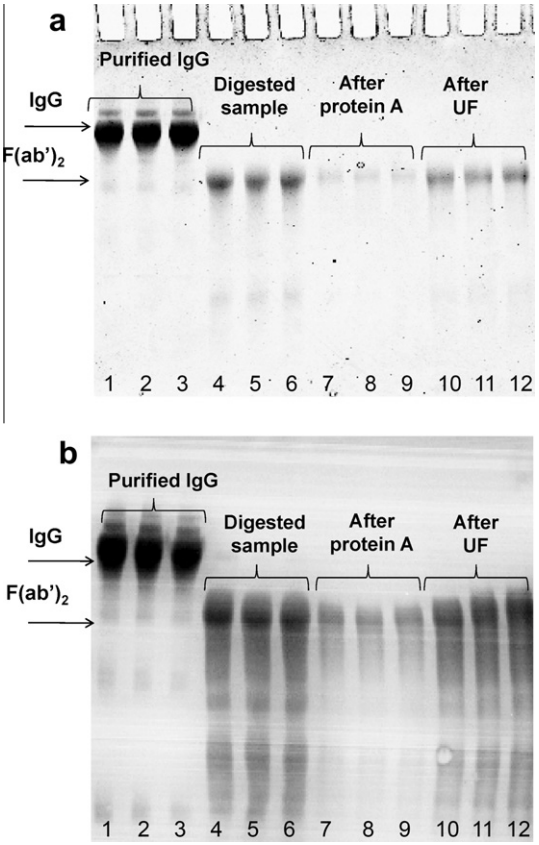

Fig. 5. SDS-PAGE (a) and Western blotting (b) of a gel run under non-reducing condition of samples from 3 batches after protein G purification (lane 1–3), peptic digestion (lane 4–6), protein A purification (lane 7–9) and ultrafiltration (lane 10–12).

at ~70% (based on UV absorbance measurements at 280 nm), which is excellent for a multi-step purification process.

Fig. 5a shows SDS–PAGE using Coomassie blue staining of the products from the different process steps. After purification with protein G affinity chromatography, non-IgG in crude plasma was removed, since only IgG bands were shown in lanes 1–3. Besides the monomer band, a small band of IgG dimers was detected. Purified IgG could be completely digested by a pepsin-to-protein ratio of 5:45 (w/w), and $F(ab')_2$ (MW ~100 kDa) was obtained (lanes 4–6). Next, protein A affinity chromatography was used to eliminate undigested IgG and Fc. As seen in lanes 7–9, the sample solutions were too diluted and only a faint $F(ab')_2$ band could be seen. However, after ultrafiltration, the samples were more concentrated, and low molecular weight impurities, obvious from the bands at ~45 kDa in the digested samples (lane 4–6), had disappeared as seen in lane 10–12.

Western blotting showed that the samples after purification with protein G and protein A including centrifugal ultrafiltration, the purified IgG (lanes 1–3), the digested samples (lane 4–6), and the purified $F(ab')_2$ (lanes 10–12) contained intact epitopes (Fig. 5b).

HP-SEC was used as additional technique to confirm the effectiveness of the new production process. The HP-SEC chromatograms of the three batches were comparable (data not shown), with the results of one batch shown in Fig. 6. The purified IgG obtained by protein G affinity chromatography eluted at 29 min and non-covalent aggregates were measured. After peptic digestion, purified IgG was broken down to give $F(ab')_2$ and small proteins/peptides. No evidence of larger aggregates was observed. Extensive purification with protein A and centrifugal ultrafiltration could remove most of the small peptides and increase the concentration of $F(ab')_2$. It can be concluded that purification of $F(ab')_2$ at a high molar recovery of about 70% can be achieved by the new process.

3.7. Characterization of purified $F(ab')_2$

For the structural characterization, the three batches of purified $F(ab')_2$ were analyzed by far-UV CD (secondary structure) and intrinsic fluorescence (tertiary structure) and the results compared to ERIG product (Fig. 7a). The three batches were comparable in their far-UV CD spectra (Fig. 7a). The far-UV CD spectra of the purified $F(ab')_2$ from three batches were similar and in good agreement with the CD spectra of $F(ab')_2$ reported by Albar et al. [23]. The $F(ab')_2$ spectra were dominated by a negative minimum around 216 nm, indicative of a high content of β-sheet structure as typically also found for IgG [26–28]. For ERIG product, a slight spectral change with a more negative ellipticity at 216 nm was measured, and also the minimum peak of ERIG product was slightly shifted to a lower wavelength at 214 nm. These results point toward slight differences in the secondary structure of ERIG product and the purified $F(ab')_2$.

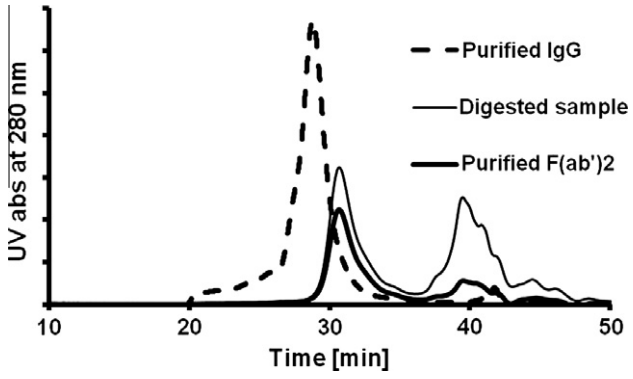

Fig. 6. HP-SEC of purified IgG, digested sample and purified $F(ab')_2$ (injection volume 50 µl) at flow rate of 0.3 ml/min.

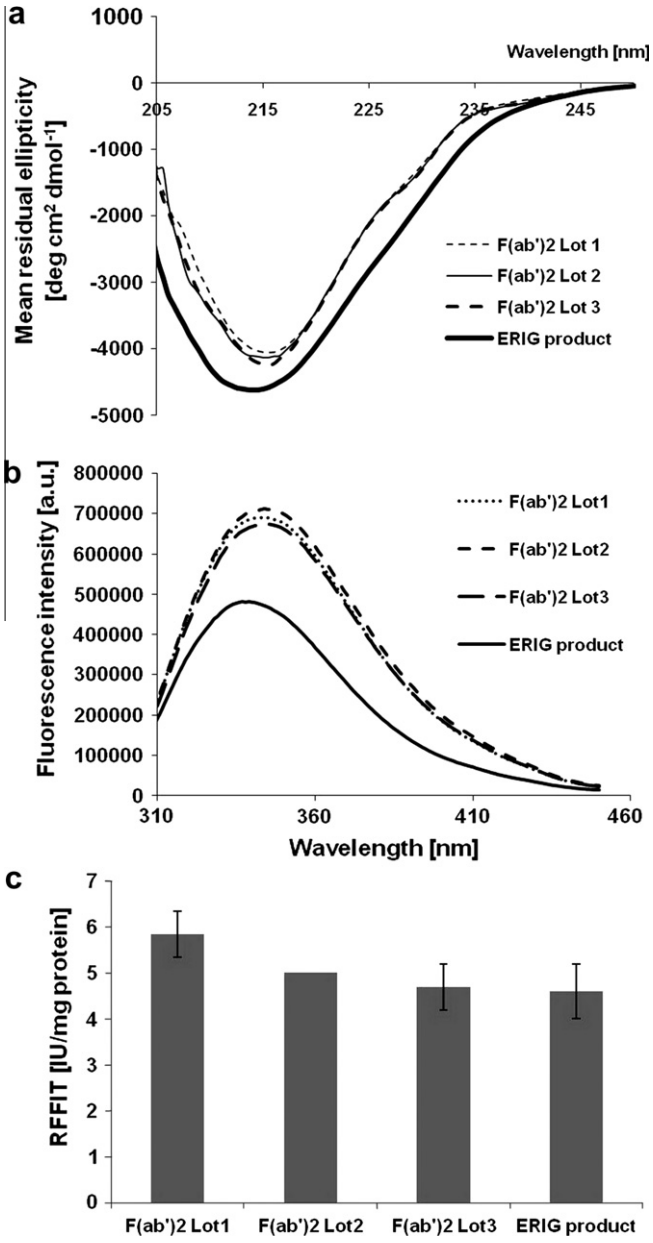

Fig. 7. Characterization of purified $F(ab')_2$ by (a) far-UV CD, (b) intrinsic fluorescence and (c) RFFIT potency.

Due to its high sensitivity, intrinsic fluorescence spectroscopy is a powerful technique to monitor structural changes of proteins, mainly in the tertiary structure [27]. The emission maximum of purified $F(ab')_2$ from three batches remained constant at 344 nm, while the spectrum for ERIG product was characterized by a decrease in the maximum intensity and an emission maximum shifted to 338 nm (Fig. 7b). Apparently, Trp residues are present in a more hydrophobic environment, e.g., more buried in the core of the molecule or in aggregates within the ERIG product.

ERIG product and the purified $F(ab')_2$ from three batches exhibited differences in the secondary and tertiary structures. To evaluate the impact of these structural differences on activity, the potency of ERIG and the three different batches of $F(ab')_2$ was measured by RFFIT (Fig. 7c). The average RFFIT potency of the purified

$F(ab')_2$ from three batches was 5.2 IU/mg protein, while that of ERIG product was slightly lower with 4.6 IU/mg protein. Overall, the purified $F(ab')_2$ from the new process obviously has a potency that is at least as high as that of the ERIG product.

Although protein G and protein A affinity chromatography can be effectively used to purify antibodies, the major limitation at production scale is cost of operation compared with precipitation techniques. In general, precipitation techniques are cheap but give low yield with an intermediate purity, whereas protein G and protein A affinity chromatography not only give higher yield and purity but also are more expensive. Therefore, the achievement of high yield and purity could compensate for the increase in the cost of the process.

4. Conclusions

This paper provides a novel process for the production of $F(ab')_2$ from crude plasma. The new process avoids salt precipitation, which is time-consuming, difficult to perform on the large scale under sterile conditions, and generally low in yield. The selected purification steps (protein A and G affinity chromatography, ultrafiltration) are routinely used in the industrial-scale production of antibody products and suitable for scale up. Introducing a purification step to isolate IgG from crude plasma was crucial for improving the efficiency of the pepsin digestion step. Most importantly, we could produce a favorably refined $F(ab')_2$ product at higher yield and slightly higher bioactivity than the current method.

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References

[1] WHO, WHO Expert Consultation on Rabies, First Report, 2005.
[2] V. Morais, H. Massaldi, Effect of pepsin digestion on the antivenom activity of equine immunoglobulins, Toxicon 46 (2005) 876–882.
[3] H.A. Hong, E.J.M. Rooijakkers, N.T. Ke, J. Groen, A.D.M.E. Osterhaus, Method for the purification of equine rabies immunoglobulin: effects on yield and biological activity, Biologicals 22 (1994) 1–6.
[4] R. Rodigues-Silva, M. Martins, A. Magalhaes, M. Santoro, Purification and stability studies of immunoglobulins from *Lachesis muta muta* antivenom, Toxicon 35 (1997) 1229–1238.
[5] R.G.A. Jones, J. Landon, Enhanced pepsin digestion: a novel process for purifying antibody $F(ab')_2$ fragments in high yield from serum, J. Immunol. Methods 263 (2002) 57–74.
[6] T. Saetang, N. Treamwattana, P. Suttijipaisal, K. Ratanabanangkoon, Quantitative comparison on the refinement of horse antivenom by salt fractionation and ion-exchange chromatography, J. Chromatogr. B: Biomed. Sci. Appl. 700 (1997) 233–239.
[7] A.J. Harms, The purification of antitoxic plasmas by enzyme treatment and heat denaturation, Biochem. J. 42 (1948) 390–397.
[8] A. Fernandes, J.Q. Kaundinya, G. Daftary, L. Saxena, S. Banerjee, P. Pattnaik, Chromatographic purification of equine immunoglobulin G $F(ab')_2$ from plasma, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 876 (2008) 109–115.
[9] R. Raweerith, K. Ratanabanangkoon, Fractionation of equine antivenom using caprylic acid precipitation in combination with cationic ion-exchange chromatography, J. Immunol. Methods 282 (2003) 63–72.
[10] W. Jiskoot, J.J. Van Hertrooij, J.W. Klein Gebbinck, T. Van der Velden-de Groot, D.J. Crommelin, E.C. Beuvey, Two-step purification of murine monoclonal antibody intended for therapeutic application in man. Optimisation of purification conditions and scaling up, J. Immunol. Methods 124 (1989) 143–156.
[11] A.D. Friesen, Chromatographic methods of fractionation, Dev. Biol. Stand. 67 (1987) 3–13.
[12] R.G.A. Jones, J. Landon, A protocol for 'enhanced pepsin digestion': a step by step method for obtaining pure antibody fragments in high yield from serum, J. Immunol. Methods 275 (2003) 239–250.
[13] H.E. Schultze, H. Haupt, K. Heide, N. Heimburger, H.G. Schwick, Comparative investigations of purified diphther and tetanus T-components and their fragments, Immunochemistry 2 (1965) 273–284.
[14] C.G. Pope, The action of proteolytic enzymes on the antitoxins and proteins in immune sera: I. True digestion of the proteins, Br. J. Exp. Pathol. 20 (1939) 132–149.
[15] C.G. Pope, The action of proteolytic enzymes on the anti-toxins and proteins in immune sera: II. Heat denaturation after partial enzyme action, Br. J. Exp. Pathol. 20 (1939) 201–212.
[16] M. Erntell, E.B. Myhre, U. Sjöbring, L. Björck, Streptococcal protein G has affinity for both Fab- and Fc-fragments of human IgG, Mol. Immunol. 25 (1988) 121–126.
[17] J. Curd, T.W. Smith, J.C. Jaton, E. Haber, The isolation of digoxin specific antibody and its use in reversing the effects of digoxin, Proc. Natl. Acad. Sci. USA 68 (1971) 2401–2406.
[18] S. Ghose, B. Hubbard, S.M. Cramer, Binding capacity differences for antibodies and Fc-fusion proteins on protein A chromatographic materials, Biotechnol. Bioeng. 96 (2007) 768–779.
[19] J. Sjöquist, Structure and immunology of protein A, Contrib. Microbiol. Immunol. 1 (1972) 83–92.
[20] L. Sjostrom, I.H. Al-Abdulla, S. Rawat, D.C. Smith, J. Landon, A comparison of bovine and equine antivenoms, Toxicon 32 (1994) 427–433.
[21] U.K. Laemmli, Cleavage of structure proteins during the assembly of the head bacteriophage T_4, Nature 227 (1970) 680–685.
[22] S. Hermeling, L. Aranha, J. Damen, M. Slijper, H. Schellekens, D.J. Crommelin, W. Jiskoot, Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon alpha2b, Pharm. Res. 22 (2005) 1997–2006.
[23] J.P. Albar, C. Juarez, F. Vivanco-Martínez, R. Bragado, F. Ortíz, Structural requirements of rabbit IgG $F(ab')_2$ fragment for activation of the complement system through the alternative pathway-I. Disulfide bonds, Mol. Immunol. 18 (1981) 925–934.
[24] A. Hawe, Structural properties of monoclonal antibody aggregates induced by freeze-thawing and thermal stress, Eur. J. Pharm. Sci. 38 (2009) 79–87.
[25] J.S. Smith, P.A. Yager, G.M. Baer, A rapid reproducible test for determining rabies neutralizing antibody, Bull. World Health Organ. 48 (1973) 535–541.
[26] B. Demeule, M.J. Lawrence, A.F. Drake, R. Gurny, T. Arvinte, Characterization of protein aggregation: the case of a therapeutic immunoglobulin, Biochim. Biophys. Acta 1774 (2007) 146–153.
[27] A. Hawe, W. Friess, M. Sutter, W. Jiskoot, Online fluorescent dye detection method for the characterization of immunoglobulin G aggregation by size exclusion chromatography and asymmetrical flow field flow fractionation, Anal. Biochem. 378 (2008) 115–121.
[28] A.P. Vermeer, W. Norde, The thermal stability of immunoglobulin: unfolding and aggregation of multi-domain protein, Biophys. J. 78 (2000) 394–404.