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Original Article

Reduction of Purification Time of Polyspecific Equine $F(ab')_2$ Antivenom against Scorpion Envenomation

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Background and Aims: In this study we improved the purification of immunoglobulins from equine antiserum raised against the venom of 6 types of scorption species. Caprylic acid (octanoic acid), a fatty acid, was found to have no effect on the activity of the enzymes pepsin, which is used in antivenom purification to digest Fc fragment of immunoglobulins to obtain $F(ab')_2$.

Materials and Methods: A new method was developed for the production of $F(ab')_2$ antivenom whereby whole equine antiserum was mixed with equal amount of 0.15 M HCl and pH 3.4 with pepsin 660 mg/L of diluted antivenom and incubated for 4 h at 37˚C. After digestion the pH were brought to 4.8 with sodium hydroxide solution (1.5 M) and then 1.5% caprylic acid and 10% ammonium sulfate was added and mixed for 60 minutes and passed through filter paper.

Results: Caprylic acid caused precipitation of albumin, and ammonium sulfate reduced turbidity of solution, resulting in a reduced protein load presented to the digestion enzymes culminating in substantial reductions in processing time.

Conclusions: The equine $F(ab')_2$ obtained using these novel caprylic acid methods were comparable in terms of yield, purity and specific activity to those obtained by multi-step and time consuming conventional salt fractionation with ammonium sulfate.

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Introduction

Antivenoms against snakes and scorpions bites have been produced worldwide for about a century and in Iran these antivenoms were produced for about 70 years ago. However, there remain various aspects of salt fractionation in conventional production process that should be optimized. For example, the, processes of fractionation should be developed to improve the recovery, potency, tolerability and stability of the product [1]. In Razi Serum and Vaccine Research Institute (South-Western Iran), the therapeutic poly-valent equine immunoglobulin, following pepsin digestion of Fc fragment from IgG, $F(ab')_2$ fragments against scorpion venoms, has been fractionated by ammonium sulfate precipitation which is a time consuming and expensive method. Recently by caprylic acid precipitation at certain concentrations, albumin and other undesired impurities are precipitated and filtered by filter paper and discarded. Filtrate containing antibody $F(ab')_2$ fraction, can be concentrated by ultrafiltration or centrifugation or precipitated with ammonium sulfate and the precipitate is dialyzed against distilled water [2]. It has been observed that the protein aggregates formed and present in the antivenom product purified by conventional methods may be responsible for the adverse reactions observed in anti-venom therapy [3]. Caprylic acid has been used to fractionate whole IgG [4] and also in the production of $F(ab')_2$ and Fab from ovine and equine sources [5]. It was first used to fractionate antivenom $F(ab')_2$ by Dos Santos et al. [6]. Caprylic acid, at about 5% (v/v), precipitates non-Immunoglobulin proteins and leaves the antibody in the supernatant. Therefore it is not necessary to re-dissolve the antibody repeatedly performed in older method, a process which also decreases antibody recovery. Another benefit of the caprilic acid fractionation is that it is likely to inactivate lipid enveloped viruses potentially transmissible from horse to human and remove pyrogenic factors [7]. Moreover, this process is superior in terms of higher yield, less production time and less protein aggregation when compared with ammonium sulfate precipitation method [4]. The antivenom plasma in the presence of high concentration of caprilic acid becomes quite turbid [8] and removal of protein precipitate by filtration leaves a turbid filtrate. Sequential precipitation of $F(ab')_2$ fragment present in turbid filtrate with ammonium sulfate has been shown to be highly effective in the laboratory and production scale purification of antibody from various animal plasma fluids [9-11]. Undesired plasma proteins were first precipitated by caprilic acid and the precipitate was removed by filtration or centrifugation after removal of impurities. The supernatant containing antibody was then precipitated by ammonium sulfate and dialyzed against distilled water. This simple and fast process could result in antibody with purity comparable to that achieved by other techniques like ion-exchange chromatography [12].

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It is interesting, therefore, to investigate the use of caprilic acid and ammonium sulfate in combination to fractionate equine antivenom F(ab´)$_2$ [13] and see whether any improvement in terms of turbidity, yield, purity and the absence of protein aggregates could be achieved by the conventional method using ammonium sulfate. Since antivenom is mainly used in developing countries where cost of production is an important factor, the manufacturing process should be simple with minimal steps and be economical as well [14].

Therefore, in this study caprilic acid and ammonium sulfate were studied at concentrations that precipitated only non-antibody plasma proteins for the fractionation of horse F(ab´)$_2$ antivenom. In order to simplify and minimize the manipulation steps involved in the fractionation, caprilic acid and ammonium sulfate were used sequentially without intermediate separation of the precipitate.

Materials and Methods

Chemicals

Lyophilized scorpion venoms of 6 species (*Hemiscorpius lepturus* belonging to *Hemiscorpionidae* family, *Androctonus crassicouda, Mesobuths eupeus, Hottentuta saulcyi, Hottentuta schach, Odonthobuthus doriae* belonging to *Buthidae* family) produced in Razi Serum and Research Institute, Ahvaz branch, Iran were used for hyper-immunization of 20 female horses. Plasma was separated from whole blood by sedimentation using sodium citrate solution as anticoagulant with added preservative [15]. Chemicals and biochemicals were of reagent grade and were from Sigma-Aldrich (St-Louis, MO, USA) or Merck (Germany).

Conditions for pepsin digestion of horse plasma

Pepsin digestion of horse polyvalent plasma was carried out at 37˚C, for 4 hours using pepsin (EC. 3.4.23.1) to plasma volume in the ratio of 660 mg per liter of initial plasma pH 3.5. The digestion was stopped by adding 1 M NaOH to bring the pH to 6.0.

Fractional precipitation of horse anti-scorpion F(ab´)$_2$ by caprilic acid and ammonium sulfate

Using a preparative format, all manipulations were carried out at room temperature of about 26-27˚C. Pepsin digested plasma (100 mL and pH 4.8) was divided into 6 parts. This study involved the fractionation of equine scorpion antivenom F(ab')$_2$ by combined stepwise ammonium sulfate and caprylic acid precipitation without intermediate separation of precipitate. Six conditions with combinations of ammonium sulfate (0-20% saturation) and caprilic acid (0-3.5% v/v), were tested. ammonium sulfate significantly reduced the turbidity raised by caprilic acid. High specific antibody activity was observed in the area containing 2-3.5% caprilic acid and 0-20% saturation with ammonium sulfate. Out of these results, 6 precipitation conditions were selected for detailed quantitative studies (Table 1). As seen in table 1, some combinations, like one with 1.5% caprilic acid and 15% ammonium sulfate and another with 3.5% caprilic acid and 0% ammonium sulfate, gave the highest fold purification (1.71 and

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1.69) with antibody recoveries at 72.79% and 77.28%, respectively.

The combinations of caprilic acid and ammonium sulfate offered a benefit over caprilic acid alone in reduction of turbidity and in promotion of purity but not the recovery of antibody. The conditions giving more favorable overall results were with 3.5% caprilic acid alone and another with a combination of 1.5% caprilic acid and 15% ammonium sulfate. These preparations of $F(ab')_2$ were homogeneous and without protein aggregate.

Ammonium sulfate at various concentrations was added slowly to each row with vigorous shaking for 60 min. The final concentration of ammonium sulfate used in this experiment was 0-20% saturation. The ammonium sulfate treating digested plasma in each beaker was adjusted to pH 4.8 with 1.5 M HCl. caprilic acid was added slowly to each container to leave final concentrations of 0, 1.5, 3.5 % (v/v) with vigorous shaking for 60 min. A condition containing digested plasma with 0% ammonium sulfate and 3.5 caprilic acid was used as the standard reference. The turbidity of each container was measured. Turbidity was again measured after the plate was kept overnight at 4°C. The contents of all the containers were transferred onto the filter papers. The filtrates were collected in the clean containers. The protein concentration and antibody activity in the filtrates were determined.

Specific antibody

The enzyme-linked immunosorbent *assay* (ELISA) antibody titer of $F(ab')_2$ of each precipitation condition was determined by a competitive ELISA. The procedure was modified as described by Morais and Massaldi [16] and Rial et al. [17]. Preparation of polyvinyl microtiter plate was according to the reported procedure [18]. Appropriate dilutions with different precipitation conditions were described [19]. ELISA plate was made according to the reported methods [20]. The units of activity in the microplate precipitation experiments were fixed with the sample dilution at 1:80 and absorbance of the sample was compared to that of the standard [21]. The unit of activity of the test tube precipitation experiment was defined as the sample dilution which had an absorbance equal to half the absorbance of conjugate alone (50% competition) [22]. The antibody unit was calculated using Prism 5, Graph Pad software program (trial version) [23].

Statistical analysis

All the tests were performed in five replications and were presented mean±standard deviation. Data were statistically analyzed using analysis of variance (ANOVA) and Duncan's multiple range test by means of SPSS (standard version 19.0 SPSS Inc, Chicago, IL, USA). There was a significant difference in the value of $p<0.05$.

Results

From the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the digested plasma protein profiles, IgG digested to $F(ab')_2$ was completely digested at 60 min, while albumin was completely digested at much shorter period in less than 15 min. Fractionation of pepsin-digested horse plasma

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using ammonium sulfate and caprilic acid precipitation under a 1000 mL format fractionation of horse plasma was performed.

Turbidity of the ammonium sulfate and caprilic acid precipitated pepsin-digested plasma: The turbidity of the precipitation conditions is shown in Table 1. In the absence of caprilic acid, an increase in ammonium sulfate concentration did not noticeably affect the turbidity. However, in the absence of ammonium sulfate, the turbidity of the suspensions sharply increased when caprilic acid was added, and at 3.5% (v/v) caprilic acid, the highest turbidity was observed.

Protein concentration of the filtrates of ammonium sulfate and caprilic acid precipitated pepsin-digested plasma: Protein concentration of the filtrates of ammonium sulfate and caprilic acid precipitated pepsin-digested plasma is shown in Table 1. At 10% saturated ammonium sulfate, protein concentration in the filtrate decreased. When caprilic acid was used, it sharply decreased the protein concentration in the filtrate; the combination of ammonium sulfate and caprilic acid resulted in more effective protein precipitation, especially at higher reagent concentrations. While the digested plasma control contained the highest protein concentration (52.25 mg/mL), the filtrate of 0% saturated ammonium sulfate and 3.5% (v/v) caprilic acid treated plasma contained the lowest protein concentration (24.86 mg/mL), indicating that 66% of the digested protein had been precipitated.

ELISA antibody titer of the filtrate of the 10% ammonium sulfate saturation and 1.5% caprilic acid precipitated pepsin-digested plasma of all the filtrates was the highest and the digested plasma control without any addition of caprilic acid and ammonium sulfate showed the highest antibody titer. This gradually decreased when ammonium sulfate and caprilic acid were added (Table 1). At 10% saturated ammonium sulfate and 1.5% (v/v) caprilic acid, the ELISA antibody titer in the filtrate was lowest, as compared to that in the digested plasma control. Specific antibody activity of the filtrate of ammonium sulfate and caprilic acid precipitated pepsin-digested plasma: The specific activity was lowest in the digested plasma control but was higher when caprilic acid and ammonium sulfate were added to selectively precipitate non-antibody proteins. At 10% saturated ammonium sulfate and 1.5% (v/v) caprilic acid, the highest specific activity, i.e, the highest degree of purification of $F(ab')_2$ was achieved.

Fractionation of pepsin-digested horse antivenom plasma using combination of ammonium sulfate and caprilic acid precipitation as studied in tubes: Six precipitation conditions which gave relatively low turbidity and high ELISA antibody titer and specific activities in the microplate experiment were selected (Table 1) for detailed quantitative studies. The turbidity (% solid) of the reaction mixtures was similar to that observed in the microplate experiment. While an increase in caprilic acid concentration resulted in increase in turbidity, ammonium sulfate was shown to decrease the turbidity caused by caprilic acid. The lowest turbidity (0.4158%) was observed

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in 20% ammonium sulfate and 1.5% caprilic acid precipitation.

The protein concentrations in the filtrates decreased significantly when precipitation was carried out at higher concentrations of caprilic acid and the presence of ammonium sulfate increased the amount of protein precipitate (Fig. 1 and Fig. 2). When the precipitation was left overnight, slightly lower protein concentration was found in the filtrates, indicating that more protein was precipitated antibody recovery. The presence of ammonium sulfate further increased the specific activity and decreased the antibody recovery. The highest fold-purification of 1.69 and 1.71 were observed at 3.5% caprilic acid and at 1.5% caprilic acid with 15% ammonium sulfate, respectively, but with antibody recoveries of only 77.28% and 72.79%. Highest antibody recoveries of 81.65% and 79.45% were observed at 10% ammonium sulfate and 1.5% caprilic acid and 20% ammonium sulfate and 1.5% caprilic acid, respectively, with 1.60 and 1.61 fold purification (Table 1). The lowest protein content was observed in the condition of 20% saturation with ammonium sulfate and 2% caprilic acid. The turbidity of the mixtures of these precipitation conditions were in the medium range of about 0.41%-0.49% which was considerably lower than that of 3.5% caprilic acid alone (0.6459%). Comparison of SDS-Page of products purified with ammonium sulfate (old method) and those purified with caprilic acid are shown in Fig. 3.

Table 1. Quantitative estimation of volume, protein, antibody specific activity, antibody recovery and turbidity of fractions obtained from precipitation at various ammonium sulfate and caprilic acid concentrations

Condition %AS	Condition %CA	Volume of Filtrate (mL)	Protein of Filtrate (mg/mL)	Total Protein (mg)	Antibody Recovery (%)	specific Activity (unit/mg)	Fold Purification	Turbidity
0	0	870 ± 5.61^{e}	52.14 ± 0.47^{d}	45.16 ± 1.09^{d}	100.00 ± 0.00^{f}	305.19 ± 0.04^{a}	1.00 ± 0.00^{a}	0.0000 ± 0.0000^{a}
0	3.5	774.6 ± 5.54^{c}	24.528 ± 0.95^{b}	19.00 ± 0.86^{b}	77.28 ± 1.10^{c}	526.93 ± 12.80^{c}	1.69 ± 0.02^{d}	0.6459 ± 0.00273^{f}
10	1.5	796.2 ± 1.64^{d}	27.764 ± 0.49^{c}	22.41 ± 0.65^{c}	81.65 ± 0.85^{e}	484.28 ± 8.23^{b}	1.60 ± 0.008^{c}	0.4957 ± 0.00213^{d}
15	1.5	725.4 ± 2.70^{b}	25.258 ± 0.39^{b}	18.3 ± 0.31^{b}	72.79 ± 0.84^{b}	$537.89+20.70^{d}$	$1.71\pm.008^{d}$	0.4545 ± 0.00219^{c}
20	1.5	794.6 ± 5.31^{d}	28.05 ± 0.87^{c}	21.96 ± 0.42^{c}	79.45 ± 0.71^{d}	493.60 ± 4.67^{b}	1.61 ± 0.007^{c}	0.4158 ± 0.00219^{b}
20	2.0	649 ± 17.63^{a}	20.00 ± 0.08^{a}	12.98 ± 0.39^{a}	59.45 ± 0.60^{a}	519.23 ± 4.78^{c}	1.52 ± 0.03^{b}	0.5819 ± 0.01206^{e}

Values are expressed as mean±SD (n=5), and values in the same line with different superscripts (a-e) are differences significant at $p<0.05$ by Duncan test using SPSS. (All of reaction voloum:1000 mL).
AS=Ammonium sulfate; CA= Caprilic acid

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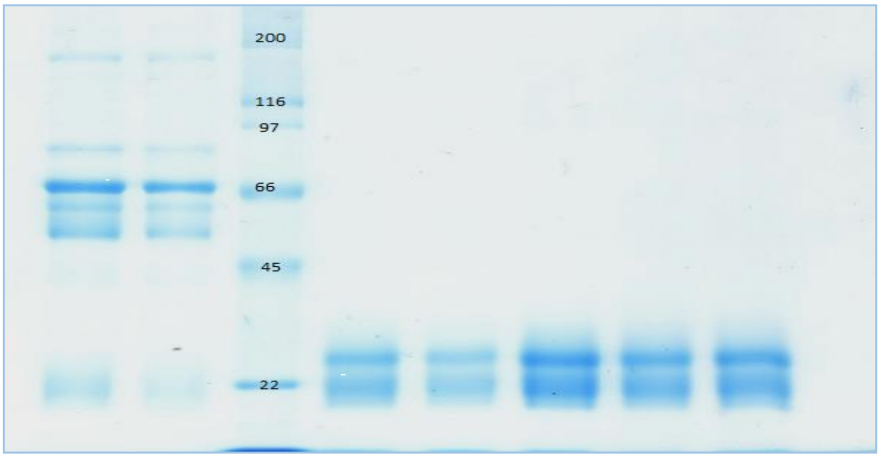

Fig. 1. SDS-PAGE of purified F(ab')$_2$ fractionated with ammonium sulfate and caprilic acid at various concentrations. The electrophoresis was carried out in 10% acrylamide under reducing condition.

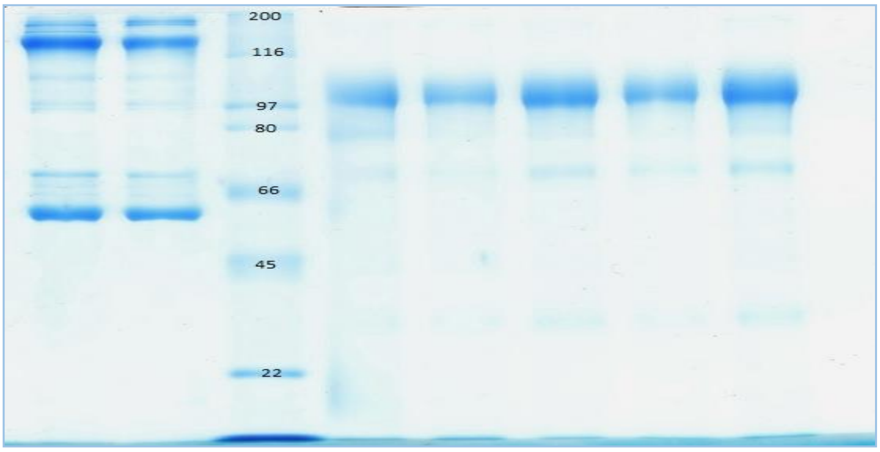

Fig. 2. SDS-PAGE of purified F(ab´)$_2$ fractionated with ammonium sulfate and caprilic acid at various concentrations. The electrophoresis was carried out in 10% acrylamide under non-reducing condition.

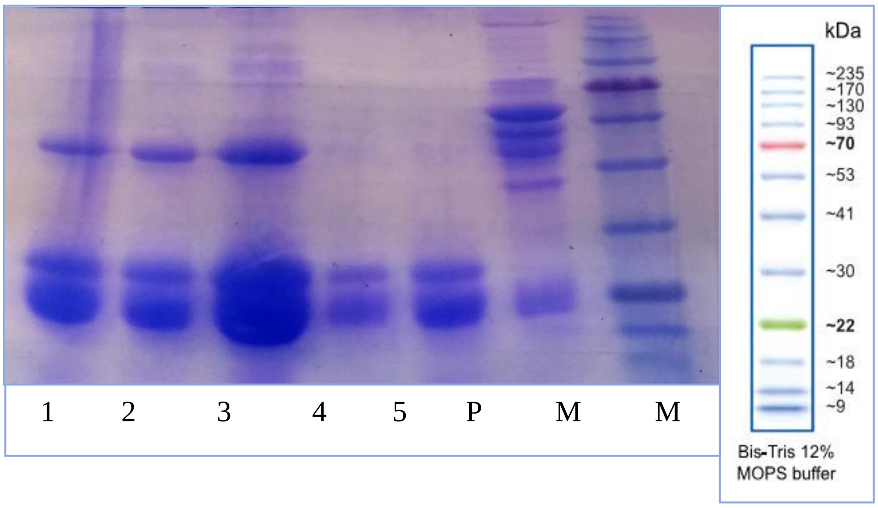

Fig. 3. Comparison of scorpion plasma purification. Lanes **1**, **2** and **3**: Experimental purification with ammonium sulfate alone (old method), **4** and **5**: Experimental purification with caprilic acid and ammonium sulphate, **P**: Plasma, **M**: Marker.

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Discussion

Large scale production of therapeutic antivenom against scorpion sting generally includes pepsin digestion of equine hyperimmune plasma and then fractionation of the F(ab´)$_2$ fragment with ammonium sulfate. [22]. Intravenous administration of antivenoms is connected with a high (10-76%) prevalence of negative effects [3]. Additional contaminants considered to result in negative effects consisting of serum proteins and other fragments or excessive molecular weight aggregates. Consequently, fractionation of antivenom antibody has aimed at clearing the Fc portion by pepsin digestion and also eliminating additional plasma protein impurities or their digestion products by numerous procedures [18,19]. Pepsin digestion has been utilized since 1939 to eliminate the greatly immunogenic Fc of heterologous antibody. Nevertheless, it is essential for the pepsin to be entirely inactivated or eliminated in subsequent fractionation procedures because the enzyme can influence the stability of the antivenom [14]. Ammonium sulfate is commonly used in large-scale fractional precipitation of IgG or F(ab´)$_2$. The negative aspect of ammonium sulfate precipitation is usually that the F(ab´)$_2$ product needs to be recovered and resolubilized. This method is hard to perform on a large scale under aseptic situations, and endproduct pollution is usually discovered. The procedure can also involve an important decrease of antibody activity [1]. Incorporation of caprilic acid with ammonium sulfate fractionation has the benefit of precipitating numerous serum proteins or their fragments. Considerably, the high molecular weight aggregates were not seen in the F(ab´)$_2$ end product purified by caprylic acid precipitation. The risk of viral contamination of biological products is a subject of great interest [7]. In this regard, the fractionation studied regarding pepsin digestion and also treatment with caprylic acid might inactivate several viruses because of the acidic situation and the detergent action of the organic acid [22]. Since the purification of antibody is generally performed by dialysis in cellulose bags, this procedure is time-consuming and can take several days. With regards to the above notes, the aim of the present study was to prepare equine antivenom using combination of caprilic acid and ammonium sulfate methods as a novel and effective combinative method in improving of F(ab´)$_2$ antivenom.

Conclusion

The present study made an attempt to purify equine antivenom using combination of caprilic acid and ammonium sulfate fractionation method in improving pepsin digested horse F(ab´)$_2$ antivenom. The results of this study showed that using caprilic acid and then ammonium sulfate can be effective in removal of impurities and extraction of F(ab')$_2$ at high volumes in shorter period of time, which is an important factor in production of biological products. we used this method for purification of 50 liters of hyperimmune

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plasma. The most important benefits of this method are 1) cheaper production cost which is an important factor in poor developing countries, 2) The shorter purification time which is the most important factor in biological product purification, and 3) removal or inactivation of progeny and protein aggregation and viral. Our results was consistent with those of other researchers who asserted this method as effective in antivenom large scale purification.

Conflict of Interest

The authors declare that they have no conflict of interest.

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