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Purification of Therapeutic Serums of Snake Anti-Venom with Caprylic Acid

Nilofar Norouznejad[1], Hossein Zolfagharian[2]*, Mahdi Babaie[2], Maryam Ghobeh[1]

[1]Department of Biology, Faculty of Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran

*Corresponding Author
Hossein Zolfagharian
Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj 3197619751, Iran
Tel: +98-2634570038
E-mail: hosseinzolfagharrian@yahoo.com

Objectives: Antivenom serums have been used extensively for over a century and are the only effective treatment option for snake bites and other dangerous animal envenomations. In therapeutic serum centers, a wide range of antivenoms is made from animal serum, mainly equine and sheep, that are immunized with single or multiple venoms. This work aimed to use caprylic acid (CA) to purify therapeutic snake antivenom.

Methods: Plasma was obtained from equine immunized with a mixture of venoms. Immunized plasma was obtained by precipitation of different concentrations (2-5%) of CA. This methodology was compared to that based on ammonium sulfate (AS) precipitation. Sediment plasma proteins were purified by ion-exchange chromatography. Protein assay, SDS-PAGE, and agar gel diffusion were performed.

Results: The total protein precipitation with AS was higher than precipitation with CA, but the best results were obtained when CA was added to the plasma until a final CA concentration of 5% was reached. Chromatography and electrophoresis indicated a stronger band for the 5% CA, and the gel diffusion assay showed antigen-antibody interaction in the purified serum.

Conclusion: The use of CA compared to the routine method for purifying hyperimmune serums is a practical and cost-effective method for preparing and producing therapeutic serums. It constitutes a potentially valuable technology for alleviating the critical shortage of antivenom in Iran.

Keywords: antivenom serums, caprylic acid, ammonium sulfate, ion exchange chromatography, gel diffusion

INTRODUCTION

Snake bite mortality and the hazards of snake venom are serious public health problems worldwide [1]. Although many attempts have been made to treat snake bites, over 5 million snake bites occur worldwide each year, resulting in more than 125,000 deaths. According to published statistics, Asia has the highest number of snake bites. Although there are no exact statistics on snake bites in Iran, it is estimated that 10,000 incidents of snake bites occur each year [2]. Many studies have shown that snake venom contains complex biological, chemical, and pharmacologically active components, including proteins, peptides, enzymes, and non-protein compounds [3-6]. Due to the fatal effects and complications caused by snake bites on the different body systems, researchers have conducted extensive studies on various types of snake venom. The World Health Organization (WHO) recommends that the most effective treatment for envenomation is an antivenom serum [6].

The transfer of serum antibodies to treat or prevent snake bites is called serotherapy. Serum from animals that have developed immunity to certain protein toxins, serum from individuals who have been vaccinated against or naturally afflicted with infectious diseases (the convalescence period), or concentrated and purified immunoglobulins from healthy people are utilized in this process. Therapeutic serums are the primary treatment pillar against snake bite venom, and currently, the preparation

and production of therapeutic serums have significant global standing [7]. Given the high prevalence of snake bites in Iran and the consequent increase in mortality, the development of anti-snake venom serum has become critical. However, it takes a relatively long time to produce these serums, and the production costs are high.

Consequently, optimization of serum purification is economically and therapeutically important. Ammonium sulfate (AS) is conventionally used in purifying therapeutic serums, but this method causes product loss and protein aggregation and is a time-consuming process [8].

The use of raw heterologous serums (blood plasma of immunized animals) can cause fatal toxicities in humans. To address these issues, researchers have developed procedures for manufacturing anti-snake venom serum over time causes only minimal adverse effects in humans [9-11].

The Fc fragment of the IgG antitoxin is mainly responsible for the toxic reactions related to the induction of this serum. Therefore, IgG is enzymatically digested in many countries using pepsin into the two fragments, F(ab)$_2$ and Fc [12]. The antitoxin production process must be specific and cost-effective for emergencies such as snake bites in developing countries. The current methods for manufacturing hyperimmune antitoxins from animal plasma are of high quality, but their production costs are also relatively high. Methods based on IgG or F(ab)$_2$ precipitation with AS result in product loss and protein aggregation. Conversely, it has been reported that very rich and highly efficient IgG is obtained using caprylic acid (CA) for the precipitation of hyperimmune equine plasma (HEP) in antitoxin production [13].

Currently, increasing the quantity and quality of therapeutic serums is critical to achieving the goal of immunizing humans against diseases. Compared to antitoxins produced by using AS, those produced by employing CA cause fewer adverse reactions in humans [14]. This research examined the use of CA in producing anti-snake venom serums in the quest to improve serum quality and reduce its production costs and compared them with those prepared by the traditional method involving AS.

MATERIALS AND METHODS

1. Hyperimmune serum preparation

HEP (with a mixture of equal parts of snake venoms (was provided by Razi Serum and Vaccine Research Institute Karaj,

Iran. Aliquots of 1,600 mL of equine plasma were obtained. The HEP was stored at 4℃ until purification. Equal volumes of serum (800 mL) were precipitated with CA and AS (Code of ethics committee: IR.RVSRI.REC; Date: 29 November 2020).

2. CA precipitation

Aliquots of 800 mL of HEP were adjusted to pH 3.2 with 1 M hydrochloric acid (HCl). Based on plasma volume, pepsin was added to the plasma (5 g of pepsin per liter of crude plasma, i.e., 4 g of pepsin was dissolved in 40 mL of distilled water, and the pepsin solution was added to 800 mL of plasma). Digestion was carried out for 1 h at 30℃. After 1 h, the temperature of the plasma containing pepsin was lowered to 20℃. The digestion was stopped by adding 1.5 N sodium hydroxide (NaOH) to bring the pH to 5.5. After digestion, the pH of the slurry was raised to 4.8 with 1 N NaOH, and CA was added slowly to the reaction mixture in different concentrations (2.0%, 3.0%, 4.0%, and 5.0%). The mixture was agitated vigorously for 1 h at 25℃. After 1 h, the treated plasma was poured into a falcon tube and centrifuged for 15 min at 3,000 rpm at 25℃. The precipitate was discarded, and the supernatant was filtered using ultrafiltration (cutoff = 10,000 dalton). The filtered liquid was collected [15].

3. AS precipitation

Aliquots of 800 mL of HEP were diluted with distilled water (ratio 1:3). The pH of the diluted plasma was adjusted to 2.3 with 1.5 M HCl, and digestion was carried out with 10% HCl and 0.5% pepsin for 1 h. The digestion was stopped by adding 2 N NaOH to bring the pH to 4.0. The plasma sample was precipitated by adding 14% saturated AS for the initial preparation of IgG. The required amount of the salt was dissolved slowly in the plasma while stirring for 2 h at 4℃. The precipitate was discarded, and the supernatant was collected. The supernatant was precipitated by adding 18% saturated AS for 1 h with constant stirring. The precipitate was dialyzed against phosphate-buffered saline (PBS) overnight and was filtered. The above solution was then treated with different concentrations of saturated AS (up to a final concentration of 55%) at 4℃. The precipitate was collected and dialyzed under the same conditions. After removal of AS from the plasma (antiserum), the product was isotonized by adding 8 g/L of sodium chloride (NaCl) and 20 g/L of phenol [16].

4. Protein concentrations and molecular weight

The protein concentrations were determined by the Lowry method using bovine serum albumin as standard [17]. Protein samples obtained from the precipitation were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method previously described using a 12% polyacrylamide gel under non-reducing conditions. The gel was stained with Coomassie blue [18].

5. Ion-exchange chromatography

The serum treated with 5% CA was further purified by ion-exchange chromatography. The ion-exchange column (diethylaminoethyl cellulose) was equilibrated with three volumes of 0.05 M PBS (pH 7.0). The dialyzed sample was centrifuged at 3,000 rpm to clear the precipitated proteins. The supernatant was loaded into a column and eluted with 0.05 M PBS and a linear gradient of NaCl concentration from 0.0 to 0.3 mM. Purification was performed at 4℃ with a flow rate of 20 mL/h. The column was regenerated by passing through 2-3 column volumes of PBS containing 1 M NaCl. Each fraction was dialyzed for 10 h against 10 volumes of distilled water. Concentration was performed using polyethylene glycol 6000 (PEG) at 4℃ [3].

6. Gel diffusion test

The gel diffusion test was performed using 1.5% agarose gel to study the antigen-antibody interaction. Agar (0.75 g) was dissolved in 0.01 M PBS (50 mL, pH 7.0), the solution was boiled using an electric heater, and the agar was dissolved completely until transparent. Then, 8 mL of agar was poured into 60 mm Petri dishes, and the gel was allowed to solidify. Three wells were used (one in the center and one on either side). Polyvalent snake venom (50 μL) was poured into the central well, 50 μL

Table 1. Protein concentrations of plasma and serums precipitated with CA and AS

Sample	Protein concentration (g/dL)
Plasma	15.3
2% CA	5.8
3% CA	6.5
4% CA	10.3
5% CA	11.3
AS	48.7

of the unpurified plasma was poured into one of the other two wells, and the serum precipitated with 5% CA was poured into the third well. Finally, precipitin bands were studied [19, 20].

RESULTS

1. Protein concentration of plasma and precipitated serums

The amount of protein in the plasma containing CA and AS was determined by the Lowry method (Table 1).

2. SDS-PAGE

HEP was precipitated with AS and different concentrations of CA. The obtained precipitate was studied using electrophoresis to evaluate the degree of purity (Fig. 1). The amounts of proteins in the serum purified with 5% CA were significantly higher than in the other precipitated serums, and the bands were darker and clearer.

3. Ion-exchange chromatography

The serum purified with 5% CA was selected for ion-exchange chromatography based on the electrophoresis protein profile. Four peaks (fractions) were observed when 5% CA chromatography was carried out (Fig. 2).

These protein peaks were subjected to 12% SDS-PAGE. Only
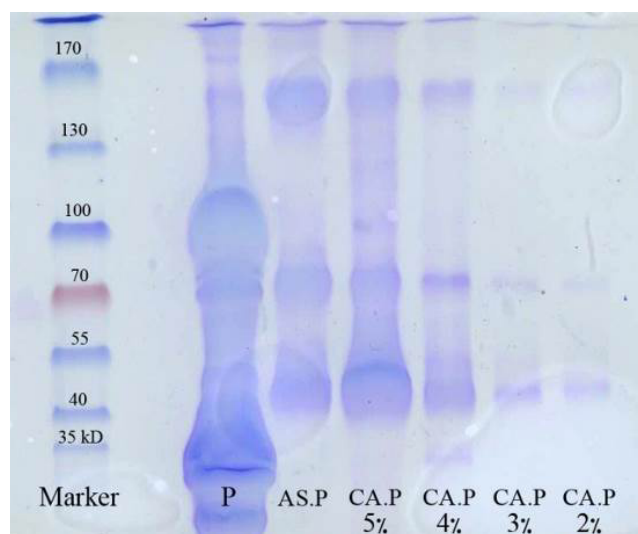

Figure 1. SDS-PAGE of precipitated HEP with AS and different concentrations of CA. P, Plasma; AS.P, Precipitated plasma with AS; CA.P, Precipitated plasma with CA.
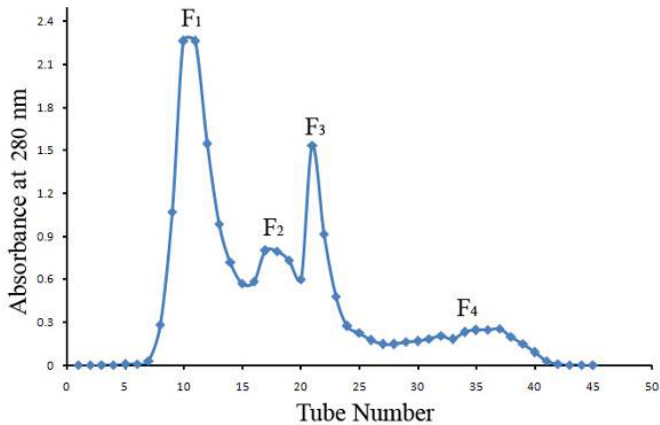
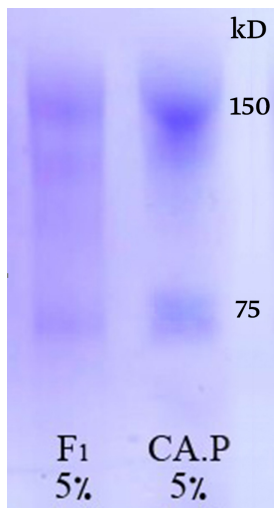**Figure 2.** Chromatogram of precipitated HEP with 5% CA.

Figure 3. SDS-PAGE of 5% CA and first fraction. CA.P, Precipitated plasma with CA; F_1, First fraction from ion exchange chromatography.

Figure 4. Gel diffusion. 1: Polyvalent snake venom, 2: Serum precipitated with 5% CA, 3: Unpurified plasma.

higher precipitation efficiency than the method using AS due to the lower protein concentration.

5. Gel diffusion

The gel diffusion assay and comparison of the antigen-antibody reaction of the purified plasma with the venom revealed that the serum purified with 5% CA and the plasma formed a precipitin line in the gel, and antigen-antibody interaction was observed in the purified serum (Fig. 4).

DISCUSSION

Snake bite mortality and its hazards are significant public health concerns worldwide. Many attempts have been made to treat snake bites and their effects, particularly in countries where the problem is prevalent [21]. Considering that snake bites worldwide (mainly from Asia and Africa) have led to many deaths, it is necessary to conduct studies on producing anti-snake venom serums [2].

According to the WHO guidelines, the central pillar in snake bite treatment is anti-snake venom serums. Currently, the preparation and production of therapeutic serum products have significant global standing. The increased snakebite incidence and associated mortality in Iran have made the production of anti-snake venom serums essential. Since developing such serums takes a long time and the development is very costly, optimizing their purification has economic and therapeutic significance.

the first peak showed purified IgG with two molecular weights (conventional and heavy chain IgG), and the other peaks revealed non-IgG proteins. The first fraction was subjected to 12% SDS-PAGE, and two major bands of 150 and 75 kDa were noted. The first fraction at 5% CA had the highest protein concentration (0.3 mg/mL) (Fig. 3).

4. Precipitation with AS and CA

Plasma precipitation with 5% CA had the highest efficiency. However, the filtration was extremely slow when working with CA concentrations lower than 5%, and the yield decreased. This research indicates that the protein content in the purified serum using the CA method was lower than that in the serum purified using the AS method. However, the method using CA had a
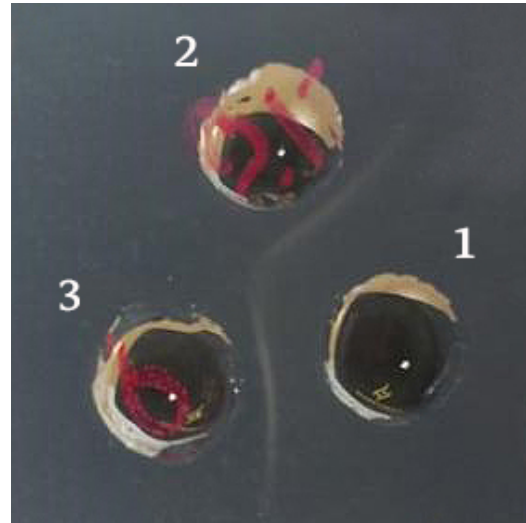
Therefore, the production of anti-snake venom serums requires consideration of commercial and economic viewpoints and epidemiological, biochemical, and immunological aspects [8].

Shortage and lack of effective anti-snake venom serums for treating snake bites in various parts of the world remains a sensitive health issue worldwide. This crisis is most severe in Africa and Asia, where effective and available anti-snake venom serums are in short supply. The production of anti-snake venom serums is a complicated process. It is essential to obtain suitable snake venom for producing hyperimmune plasma. Furthermore, the low number of producers and the susceptibility of production systems in developing countries make the provision of efficient anti-snake venom serums precarious in areas where snake bites are common [22].

Earlier, anti-snake venom serums, which contained pure equine serum, were precipitated with AS. Subsequent improvements in the technique included fragmentation of IgG molecules using pepsin to develop F(ab)₂ serums and, recently, the use of papain to generate Fab fragments [23].

In addition to being time-consuming, the use of AS causes product loss and protein aggregation in the serum. However, the use of CA saves time and causes less product loss. There are fewer stages in the purification process of therapeutic serums when CA is used instead of AS [13]. Therefore, the method proposed in this research for producing antitoxins is based on the purification of IgG antibodies through the precipitation of HEP using CA. This simple and inexpensive method for preparing therapeutic serums has many advantages [24].

In the study on equine plasma containing antibodies against *Crotalus durissus terrificus*, dos Santos et al. [25] showed that serum precipitation with CA allows obtaining very efficient IgG fragments rapidly with high levels of neutralizing and purifying activities. These results were confirmed in the present study.

Gutiérrez et al. [26] reported that hyperimmune plasma precipitation with CA is a simple, convenient, and inexpensive method for producing high-quality IgG. They showed that the purification of these serums with CA yielded better results and could effectively improve the snake bite crisis in Africa [26]. These findings are consistent with our results.

CA precipitation is a purification step that can improve the purity and quality of the antivenom. CA has recently been used to precipitate all proteins, except for IgG, in hyperimmune serums, and absorption or ion-exchange chromatography is used to further purify anti-snake venom serums. Various producers use a combination of these techniques and do not follow the same production method [15].

As mentioned above, the use of CA to precipitate HEP in producing antitoxins results in the production of very rich and highly efficient IgG [13]. In 2015, a study was conducted to purify IgG in equine serum using CA, and IgG recovery was 91-95%. This research also showed that the use of 5% CA was the most efficient, and the first fraction had the highest protein content [27].

Nudel et al. (2012) analyzed various concentrations of CA in equine plasma precipitation. They concluded that the best concentration of this acid for this purpose was 3% at pH 4.9 and 37℃ and stated that some of the immunoglobulins remained in the solution when lower concentrations of CA were used [13]. This research achieved the highest efficiency by using 5% CA.

The protein content in the serum purified using the CA method was lower than that in the serum purified using the AS method. However, given the low protein content, the precipitation efficiency was higher with the CA method than with the AS method. Based on the guidelines published by WHO for heterologous therapeutic serums, the lower the final protein content of the product, the higher the quality.

CONCLUSION

Compared to the routine method for purifying hyperimmune serums, the use of CA is a practical and cost-effective method for the preparation and production of therapeutic serums. The serum purified with 5% CA yielded the best results in the current study and had the highest efficiency among all the serums purified with different concentrations of this acid. CA precipitation is an extra purification step that can improve the purity and quality of the antivenom. Appropriate manufacturing steps in the purification processes on antisera (such as CA) can reduce the incidence of adverse reactions.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ORCID

Nilofar Norouznejad, https://orcid.org/0000-0003-3087-1707
Hossein Zolfagharian, https://orcid.org/0000-0002-8093-2400
Mahdi Babaie, https://orcid.org/0000-0001-8366-5929
Maryam Ghobeh, https://orcid.org/0000-0002-6895-6231

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