Tropical Journal of Pharmaceutical Research March 2018; 17 (3): 409-414
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i3.4

Original Research Article

Optimization of purification procedure for horse F(ab´)2 antivenom against *Androctonus crassicauda* (Scorpion) venom

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Sent for review: 29 September 2017 *Revised accepted: 25 February 2018*

Abstract

Purpose: *To immunize antiserum of horse with Androctonus crassicauda scorpion venom in order to achieve an antivenom with higher purity by combined caprylic acid/ammonium sulfate.*
Methods: *The optimum pH to terminate enzymatic digestion was evaluated. Purification was performed by various combinations of caprylic acid (0 to 2.5 mL %) and ammonium sulfate (0 to 20 g %) at 25, 30 and 37 ºC. The effects of three factors (caprylic acid, ammonium sulfate and temperature) were evaluated based on precipitation of non-immunoglobulin proteins. Antivenom purity was evaluated by determining the concentration of desired soluble protein and undesired albumin, as well as by turbidity and titration.*
Results: *The results showed that the optimum pH for inhibition of enzyme activity and precipitation of impurities was 4.8. SDS-PAGE revealed that the highest impurity precipitation and lowest protein aggregation was occurred at the combination of 1.5 mL % caprylic acid and 10 g % ammonium sulfate at 37 ºC.*
Conclusion: *The modified method of purification significantly decreases turbidity, albumin impurity concentration and processing time but increased antibody titer and purity of antivenom. Therefore, it is a potentially suitable method for purifying antivenom in commercial production.*

Keywords: *Antivenom, Scorpion, Androctonus crassicauda, Purification, Albumin, Antiserum*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Scorpion stings are considered as one of the major public health problems in many developing tropical and subtropical countries. Some studies indicated that the severity of scorpion stings varies in different regions and countries due to the existing species in geographical area providing health services [1,2]. Scorpion envenoming usually starts with local pain at the site of sting and may progress to immense clinical consequences, morbidity and mortality

[3].

The severity of scorpion envenomation symptoms varies according to scorpion type, time of sting site and age and health status [4]. Nowadays, the use of anti-venom is regarded as the best way for treating envenomed patients. Thus, some studies should be conducted to adapt new and effective ways to produce more specific and purified anti-venom for treating scorpion victims. Anti-venom is the only way to treat patients. The first anti-venom was prepared for human use by Calmette at the beginning of the Eighteen Century [5,6]. Anti-venom caused serious reactions and was sometimes as dangerous as the venom itself [7]. In order to overcome these problems, some improvements have been made in anti-venom production [8]. The methods which are currently used by all anti-venom producers, are based on heat denaturation and salting out to precipitate of specific protein with or without enzyme digestion for isolating and concentrating anti-venom antibodies. Pope and colleagues pioneered to use the salt precipitation technique [9,10]. Anti-venom is obtained from hyper immune animal serum. The whole molecule immunoglobulin G is responsible for an immune response of serum. Many researches indicated that Fc fragment lead to anaphylactic shock among patients. Thus, the manufacturer cleaves Fc fragment either through pepsin producing F (ab′) $_2$ anti-venom or papain creating Fab anti-venom. Based on the results, F $(ab)_2$ is better than Fab in neutralization and plasma distribution. Researches in Brazil showed that IgG performs more efficiently than Fab in neutralization [11]. Scientists used ammonium or sodium sulfate to precipitate immunoglobulin from immunized plasma. Although salt precipitation methods leads to high yields but contamination may occur with other proteins, such as albumin and digested Fc fragment [12].

Finally, caprylic acid has been implemented for the precipitation of albumin and other impurities. In addition, it is generally used alone or in combination with salt precipitation in order to purify immunized plasma from both equine and ovine origins [13]. Furthermore, caprylic acid is an unsaturated fatty acid and a good precipitating agent for undesired proteins from animal plasma essentially albumin without any effect on immunoglobulin fraction [14]. Thus, producers can implement caprylic acid in the present method for a successful purification as the replacement for the old process based on precipitation with ammonium or sodium sulfate [15]. Iran is very rich in arthropods especially scorpions due to its climate and weather [16]. Iran has been known as the second country after Mexico due to the presence of more than 50 species of dangerous scorpion. *Androctonus crassicauda* is considered as the second medically important scorpion's distribution in Iran [17]. Therefore, this study aimed to optimize the purification process for monospecific plasma against the venom of *Androctonus crassicauda* venom.

EXPERIMENTAL

Anti-venom

The anticoagulated blood of five female horses was immunized with *Androctonus crassicauda* venom and purchased from Razi Serum and Vaccine Research Institute Ahvaz, Iran. Then, plasma was separated from blood after sedimenting blood cells without adding any preservatives and stored at 4 ºC until purification [18]. Pepsin (EC: 3.4.23.1) and chemicals were purchased from Merck Company, Germany.

Enzymatic digestion of Fc fragment

In the first stage, monovalent horse plasma was diluted against *Androctonus crassicauda* venom with an equal volume of 0.1 M HCl. Then, the pH of the diluted plasma was adjusted to 3.5 with 1 M HCl. Pepsin (660 mg of pepsin for each liter of plasma) was added and incubated at 37 ºC for four hours. In this step, Fc fragment of IgG was broken and separated from F $(ab')_2$. Finally, in order to determine the best inhibition of enzyme activity, the solution was divided into 3 equal parts and the pH of first, second and third parts was adjusted to 4.8, 5 and 5.5 by addition 1 M NaOH, respectively.

Removal of digested proteins and impurities

Caprylic acid concentration, ammonium sulfate concentration and temperature were considered as variables of this study. The filtrate which had best pH from enzymatic digestion step, was selected for this step. Then, the temperature was brought to the specified value. In the next step, ammonium sulfate was gently added to vigorous shaking for 1 h. Thereafter, caprylic acid (octanoic acid) was added to reach the allotted concentration under vigorous stirring for 60 min. The pepsin and impurities were removed and discarded by filter paper (Whatman no. 1) and finally, the filtrate containing F $(ab')_2$ fragments was collected. Further, the concentration of protein and albumin, as well as the amount of turbidity and anti-venom antibody titer were estimated and recorded. The best obtained method was compared with those of

conventional method which is performed by ammonium sulfate.

Determination of protein concentration

Albumin concentration was determined by Bromocresol green method [19]. Protein concentrations were determined by Bauer method. In this technique, peptide bonds in proteins produce a violet color in the presence of alkaline copper and the intensity of color is estimated with spectrophotometer at 540 nm wavelength [20].

Measurement of turbidity

The turbidity of each tube was estimated by spectrophotometer at 400 nm wavelength against distilled water as blank.

Quantitation of anti-venom antibody titer

The *Androctonus crassicauda* antibody titer was estimated by ELISA described by Van Dong *et al* [21]. Then, the sample dilution (shown Table 2) was prepared and transferred to venom coated wells then, the plate was washed. Horseradish peroxidase-conjugated anti-horse IgG diluted in PBS (50 µL/well) was added to each well and incubated for 2 h at the favorable temperature. IgG of horse was used against scorpion venom as the reference standard in all ELISA plates.

Purity evaluation

The purity of product was analyzed by vertical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [22] using a 10 % polyacrylamide gel under non-reducing conditions. Then, all samples were used at the concentration of 500 µg/ml. Finally, the gel was stained with Coomassie Brilliant Blue R-250 (Bio-RA laboratories).

Statistical analysis

The data are presented as mean ± SD (n = 3). The significance of differences was analyzed by one-way analysis of variance (ANOVA), and, the level of significance was set at $p < 0.05$.

RESULTS

The inhibition of enzyme activity

pH was adjusted to 4.8, 5.0 and 5.5 with 1 M NaOH for the inhibition of enzyme activity after digestion. Figure 1 illustrates the results of SDS-PAGE which obtained pH was 4.8 as the best pH for enzyme inhibition.

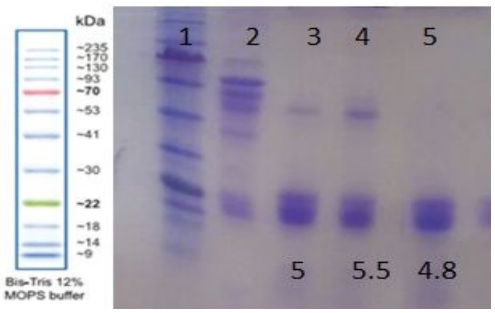

Figure 1: A comparison of pH range for inhibition of enzyme activity. (1: Plasma, 2: Marker, 3, 4 and 5: different pH for inhibition of enzyme activity)

Removed impurities and precipitated F(ab´)$_2$

Effect of concentration of caprylic acid and ammonium sulfate and temperature was evaluated on albumin and protein concentration, antibody titer and turbidity. The results are summarized in Table 1.

Results showed that an increase in turbidity resulted in increasing the concentration of caprylic acid without ammonium sulfate. In addition, an increase in temperature led to a reduction in turbidity. Significant effect was not detected on opacity in the absence of caprylic acid with increasing the concentration of ammonium sulfate. In addition, the highest opacity was observed at 25 °C and 2.5 % concentration of caprylic acid. Further, a significant reduction was observed in protein concentration indicating a more effective sedimentation of proteins, combined with caprylic acid and ammonium sulfate. The highest concentration of protein was observed in 15 % ammonium sulfate and 2 % caprylic acid, along with an increasing temperature. The presence of caprylic acid decreased the concentration of albumin because caprylic acid has binding sites for albumin leading to an increase in precipitation. The highest concentration of albumin was observed at 30 °C and with 20 % ammonium sulfate without caprylic acid.

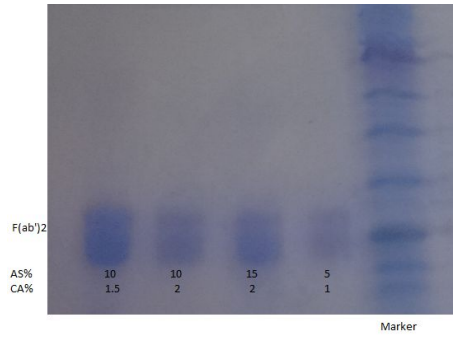

Figure 2: SDS-PAGE analysis of the purified F(ab')$_2$ fractionated with caprylic acid and ammonium sulfate in different concentrations.

Therefore, the second round of experiments was conducted through increasing the temperature and decreasing the concentration of ammonium sulfate and caprylic acid (Table 2).

Based on the summarized results in Table 2, better results were obtained for the treatment in 10, 14, 17 and 18 samples. Figure 2 shows SDS-PAGE of these four samples. The highest impurity precipitation and lowest protein aggregation were observed at combination of1.5 % (V / V) in caprylic acid and 10 % (W / V) of solid ammonium sulfate at 37 °C.

Table 1: Quantitative estimations of protein and albumin concentration, ELISA antibody titer and turbidity of fractions were obtained from fractional precipitation at various AS (0, 15 and 20) and CA (0, 2 and 2.5) concentration and temperature (25 and 30)

Sample	Condition						
	AS (g%)	***CA (mL%)***	***Temp***	***Protein concentration (mg/mL)***	***Albumin concentration (mg/mL)***	***ELISA antibody titer (unit/mL)***	***Turbidity***
1	0	2	25	29.134	0.097	12,500	0.343
2	0	2.5	25	27.745	0.117	11,900	0.387
3	15	0	25	50.846	0.099	12,011	0.000
4	20	0	25	47.578	0.124	11,410	0.000
5	15	2.5	25	21.576	0.086	12,002	0.279
6	20	2.5	25	21.789	0.091	10,520	0.158
7	15	2	25	21.765	0.074	12,110	0.263
8	20	2	25	21.154	0.087	10,640	0.138
9	0	2	30	28.654	0.089	11,965	0.312
10	0	2.5	30	25.189	0.112	11,123	0.351
11	15	0	30	49.576	0.85	11,345	0.000
12	20	0	30	47.567	0.119	10,610	0.000
13	15	2	30	19.943	0.8	11,342	0.241
14	20	2	30	19.489	0.087	9,760	0.118
15	15	2.5	30	20.643	0.069	11,870	0.197
16	20	2.5	30	19.923	0.081	10,120	0.165

Note: AS = ammonium sulfate; CA = caprylic acid; $p < 0.05$

Table 2: Quantitative estimations of protein and albumin concentration, ELISA antibody titer and turbidity of fractions were obtained from fractional precipitation at various AS (5, 10 and 15) and CA (1, 1.5 and 2) concentration, and temperature (30 and 37)

Sample	Condition						
	AS (g %)	***CA (mL%)***	***Temp***	***Protein concentration (mg/mL)***	***Albumin concentration (mg/mL)***	***ELISA antibody titer (unit/mL)***	***Turbidity***
1	5	1	30	27.612	0.097	11,511	0.219
2	10	1	30	25.432	0.117	11,421	0.234
3	15	1	30	24.334	0.099	12,234	0.249
4	5	1.5	30	25.445	0.124	11,454	0.308
5	10	1.5	30	21.743	0.086	11,516	0.166
6	15	1.5	30	21.944	0.091	12,011	0.253
7	5	2	30	24.835	0.074	11,576	0.321
8	10	2	30	23.887	0.087	11,343	0.204
9	15	2	30	22.754	0.089	12,187	0.241
10	5	1	37	25.743	0.089	10,335	0.198
11	10	1	37	23.567	0.112	10,486	0.211
12	15	1	37	22.498	0.85	11,256	0.225
13	5	1.5	37	23.676	0.119	10,286	0.289
14	10	1.5	37	19.165	0.8	10,154	0.145
15	15	1.5	37	19.889	0.087	11,176	0.233
16	5	2	37	22.776	0.069	10,454	0.314
17	10	2	37	21.489	0.081	10,244	0.187
18	15	2	37	20.276	0.076	11,269	0.201

Note: AS: Ammonium sulfate; CA: Caprylic acid ($P < 0.05$)

DISCUSSION

Scorpion envenomation is one of most important health problems among developing countries like Iran. Anti-venoms are poly or mono-specific immunoglobulin or F $(ab')_2$ fractions. The production of poly-specific anti-venom is difficult and the presence of desired protein may cause serum sickness in patients. However, mono-specific antibodies are produced against one venom and injectable proteins are reduced. In other words, an increase occurs in the specificity and potency of anti-venom and the product of anti-venom is devoid of contamination [1]. This study was performed to increase purity recovery and specific activity with proved method of purification of mono-specific anti-venom of *Androctonus crassicauda.*

Ammonium sulfate has been used for fractionation and purifying the anti-venom at final concentration of 32 % w/v for more than 100 years. In addition, it can precipitate all the immunoglobulin (IgG, IgM and IgA) from serum and leave the albumin in the supernatant. Some albumin molecules are trapped within the immunoglobulin precipitate based on the conventional method [23]. The results indicated that 4.8 was regarded as the best pH for inhibiting enzyme activity due to isoelectric pH of albumin leading to the higher removal of albumin and impure proteins. The result of the present study is consistent with those reported by Simsiriwong *et al*, proposed that pH < 7 was suitable for stopping enzyme activity [24]. The present study was conducted to increase purity, recovery and specific activity. This suggested method led to the purification of a higher volume of plasma for purifying mono-specific anti-venom against *Androctonus crassicauda* venom.

The U.S. Food and Drug Administration (FDA) and WHO have approved caprylic acid [18]. This fatty acid (caprylic acid) plays a significant role in opacity when it is used alone for removing impurities while the use of caprylic acid and ammonium sulfate together failed to produce such problems in laboratory scales. The best combination was 1.5 % caprylic acid and 10 % ammonium sulfate at 37 °C. In this study, obtained method could decrease turbidity, albumin concentration and processing time and increase yield and purity of product which are regarded as some important factors in developing countries.

CONCLUSION

The new purification technique can significantly produce anti-venom by decreasing turbidity, albumin and impurity concentration while increasing antibody titer and the purity of anti-venom. Therefore, this technique is potentially suitable for commercial production and purification of anti-venom.

DECLARATIONS

Acknowledgement

The authors are deeply thankful to Dr Amir Mohammad Kordzangene for his kind help.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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