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Production of an Efficient Enzymatically Fab Fragment Antivenom against Cobra Snake (*Naja naja oxiana*) Venom

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ABSTRACT

Since around 100 years ago, the best treatment for millions of global snakebite victims has been polyclonal antivenoms. However, common antivenoms need continuous improvement to reduce rare, their side effects and get better performance. In the present study, Fab antivenom was produced through papain digestion of anti-cobra venom plasma, multi-step purification, and optimization, including ammonium sulfate precipitation and DEAE-cellulose column chromatography. Then, the existence of the corresponding Fab fragment antibody was seen and confirmed by SDS-PAGE method and double immunodifusion (Ouchterlony) test. In addition, the potency test in NIH laboratory mice revealed that each milliliter of the new Fab antivenom was able to neutralize 624 micrograms and (80LD_{50}) of cobra venom, which is about 15% more efficient than the primary plasma of the same concentration, and 1.57 times more effective than the cobra antivenom found in commercial hexavalent antivenom of Razi Institute. According to the findings, it seems that this new Fab antivenom can be used as a new candidate for treatment of cobra snakebite victims.

Keywords: Ammonium sulfate, Cobra snake, DEAE-cellulose, Fab antivenom, *Naja naja oxiana*

1. Introduction

Snakes spread in most parts of the world, and so far, about 3,000 species have been identified, of which about 400 species are poisonous and only 50 species can be dangerous for humans. The annual number of snakebites in the world reaches 5 million, and the death rate is about 100,000 people. According to the related research, more than 21 species of semi-poisonous and poisonous snakes and 35 species of non-poisonous snakes have been identified in Iran, which cause 6,500 bites and 3-9 deaths each year (7). Although the number of casualties is not high, since the physical injuries inflicted on victims are relatively high, such bites become a real threat. Snake venom is an oily liquid with a white-to-yellow color and a slightly acidic pH. Fresh snake venom has a specific gravity of about 1.03 to 1.05, and its powder dissolves in water and normal saline solution (23). Snake venom contains a mixture of different enzymes and polypeptides and also may contain neurotoxins, proteolytic and hemolytic polypeptides, blood coagulants, phosphatases, cholinesterase, hyaluronidase, aminoacidoxidase and other enzymes and inorganic ions that stored in special glands that are equivalent to human parotid glands. At the time of the snakebite, venom is injected into the victim's body through their hollow fang's teeth. Clinically, the venoms of the elapidae snake family, such as cobras, are nerve-oriented, while, Viperidae and sea snake venoms, are blood-oriented and muscle-oriented, respectively. Nerve-oriented venoms cause paralysis, including respiratory muscle paralysis, suffocation, and other symptoms (14, 2, 24). Currently, in the Razi Institute of Iran, hexavalent antivenom containing $F(ab)_2$ antibody fragments is made against six Iranian venomous snakes, including *Naja naja oxiana*, *Vipera lebetina*, *Echis carinatus, Vipera albicornuta*, *Agkistrodon halys,* and *Pseudocerastes persicus,* which are classified into three families Elapidae, Viperidae and Crotalidae (19). Papain is a cysteine protease enzyme obtained from the white liquid (latex) of the papaya plant (*Carica papaya*). This enzyme has a high optimum temperature (65°C) and a wide pH range from 5-8 for its activity. Papain is widely used in different industries such as food, pharmaceuticals (e.g., antibody digestion), meat tenderness, and also protects plants from insects (16). For more than one century, the mainstay of primary treatment for snakebite victims has been the administration of antivenoms (5). Common methods for the production of heterologous antivenoms, require the immunization of animals such as horses, sheep, goats, and rabbits (9,12). Usually, most of the antivenoms are prepared against crude snake venoms in horses. Active components of antivenoms are antibody molecules that cause the release of blocked acetylcholine receptors in snakebite victims. These antivenoms based on their antibody type, usually classified into three groups. The first group is less common, and its antibody component is usually intact IgG molecule with a molecular weight of about 150 kDa and speed of its distribution in the body is slow and its side effects are more likely than the other two groups. The second group of antivenoms, containing $F(ab)_2$ fragment molecule with a molecular weight of about 100-110 kDa with the highest amount of production and consumption in the world. The third type of antivenoms contain Fab fragment molecule with a molecular weight of about 50 kDa. In terms of persistence in blood plasma, although IgG or $F(ab')_2$ antivenoms persist in the circulation for a longer period of time, Fab molecules due to being smaller than the other two groups, are removed sooner from the victim's body. However, Fab fragment molecules, unlike the other two molecules, have a faster spread and penetration in body tissues (1, 6, 25, 26). Due to the emergence of side effects such as serum sickness or anaphylaxis reactions along with the use of common antivenoms which may be due to protein aggregation or a few impurities (8, 19), it has always been necessary to carry out scientific work such as the current work to produce antivenoms with fewer side effects and more efficiency.

2. Materials and Methods

2.1. Digestion and purification of plasma

Fifty ml of anti-cobra venom hyperimmune equine plasma was diluted in 100 ml of PBS 1X buffer, and its pH was adjusted to 7.2. Afterward, the mixture of papain enzyme (17 mg/cc) and activation buffer was placed at 37ºC for half an hour at a temperature of 65°C for half an hour, and then the activated enzyme was added to diluted plasma at a final concentration of 3 mM and then the mixture was incubated at 37°C for 20 h. At the end of incubation, enzyme activity was stopped using iodoacetamide solution (2.8 g/l). Then, to purify the digested plasma, 50% ammonium sulfate solution was added to the plasma twice in 23% and 20% final concentration and placed on the stirrer in the incubator and laboratory temperature, respectively, and was dialyzed after each centrifugation (4025 rcf, 20°C, 20 min). Again, for more purification, saturated ammonium sulfate was added twice in 33% and 22% of the final concentration to the digested plasma and overnight incubated constantly at the refrigerator and laboratory temperature for 50 min, respectively. Same as the previous step, after each centrifugation (4025 rcf, 20ºC, 20 min.), the mixture was dialyzed using a dialysis bag (10 kDa cut-off) immersed in PBS 1X for 48 hours. Afterward, the mixture was more purified by DEAE-Cellulose ion exchange column. Finally, the Fab solution

was filtered with a 0.2 micrometer syringe filter and stored in the refrigerator for future usage.

2.2. Protein assay and SDS-PAGE test

Using the Nanodrop device, the amount of protein of Fab antivenom, the primary crude plasma and purified plasma were measured. In addition, to check the presence of 50kDa bands corresponding to the antibody Fab fragment in the new Fab antivenom, 12% SDS-PAGE gel under non-reducing condition and coomassie brilliant blue R-250 staining were used.

2.3. Ouchterlony Test

In addition to SDS-PAGE technique, Ouchterlony test was also used to check the presence of Fab fragment antibodies in the new Fab antivenom. For this, 10 ml of 1% agarose gel in PBS 1X was prepared and poured into a 55 mm Nunc plate. After cooling and hardening of the agarose gel, 5 wells were made on four sides and one in the center of the plate. Next, 100 µl of cobra venom solution was poured in the center well, and also the same amount of anti-cobra venom plasma, hexavalent serum of the Razi institute, purified plasma, and Fab antivenom were poured in the four surrounding wells, respectively. Eventually the plate was placed inside the refrigerator at the temperature of 4ºC, and the result was recorded after 96-120 h.

2.4. Potency test

To determine the neutralizing performance of the new Fab antivenom, a potency test was performed in laboratory Syrian mice. For this, seven 10 ml tubes were selected for seven groups of 18-20 g NIH laboratory mice, and the necessary materials were poured to them as presented in Table 1. The tubes were placed in the incubator at a constant temperature of 37ºC for 30 min. Afterward, 31 NIH Syrian mice weighing 18-20 grams, according to the Table 1, were randomly divided into seven groups, and the contents of each tube was intravenously injected to tail vein of mice of each group (0.5cc/mouse). Then, after placing of food and water bottle, their cages were placed in the experimental animal room with proper temperature (22°C) and ventilation, and after 24 h, the number of dead and alive mice in each group were recorded.

3. Results

3.1. Digestion and purification of plasma

The digested and purified plasma were dialyzed twice, and SDS-PAGE test was performed to check the presence of a 50 kDa band corresponding to the Fab fragment antibody on the new Fab antivenom (Figure 1). According to Figure 1, although the 50 kDa band corresponding to the Fab fragment is not present in the one-twentieth diluted crude plasma (lane 2), it is clearly seen in the produced Fab antivenom (lanes 3 and 4).

3.2. Protein assay

The total protein of monovalent crude anti-cobra venom plasma, the purified plasma, and the new Fab antivenom were measured using a Nanodrop device, and the results are illustrated in Table 2.

3.3. Ouchterlony test

To check the presence of Fab fragment in the new Fab antivenom, Ouchterlony plate was prepared and seen after 96-120 h of incubation in refrigerator. As shown in Figure 2, the wells No. 1, 2 and 3, that contained bivalent antibody molecules, showed a clear precipitation band, especially the crude plasma and hexavalent serum (wells No. 1 and 2), which have formed stronger precipitation

Table 1. Study mice groups and related injections

No.	group	injection solution mixture				total volume (ml)	No. of LD_{50}
		normal saline (ml)	Anti-cobra* venom plasma (ml)	cobra venom (1mg/cc) (ml)	Fab antivenom (ml)		
1	neg. cont.	2	-	-	-	2	-
2	neg. cont.	1	1	-	-	2	-
3	pos. cont.	2.883	-	0.117	-	3	5
4	comparative cont.	0.727	1	0.273	-	2	35
5	comparative cont.	0.688	1	0.312	-	2	40
6	Fab antivenom (test)	2.766	-	0.234	3	6	10
7	Fab antivenom (test)	0.564	-	0.936	1.5	3	80

*Hyperimmune monovalent plasma, which produced in horse against cobra snake venom.

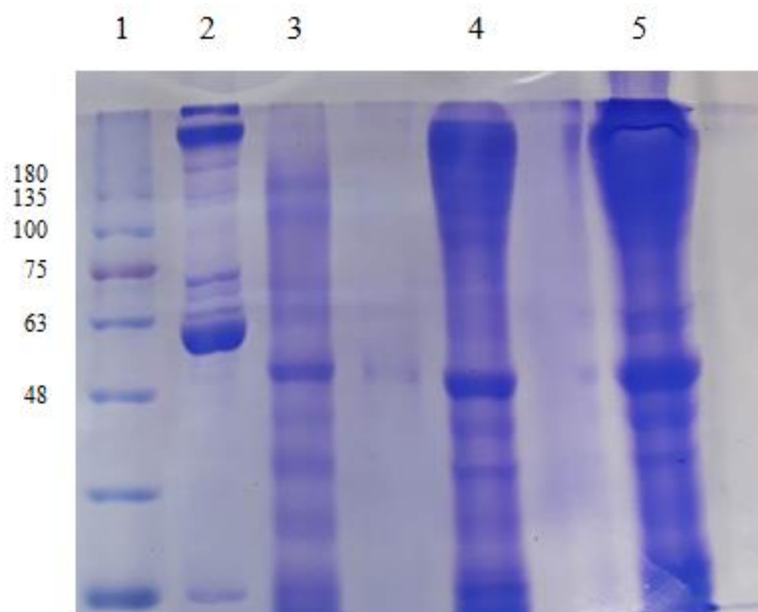


Figure 1. SDS-PAGE image of equine plasma and Fab antivenom: lane 1 protein marker; lane 2, hyperimmune monovalent crude equine plasma against cobravenom with a dilution of one twentieth; lane 3, Fab antivenom; lane 4, concentrated of sample 3. As can be seen in lane 2, the main band in the range of 60kDa corresponds to albumin, a clear band is seen, but the 50 kDa band is not seen. However, in samples 3 and 4, in the range of 50kDa, corresponds to the Fab fragment antibody, a 50kDa band is clearly visible. Lane between 3 and 4 is empty.

Table 2. Protein measurement of the samples

Row	Sample description	Protein content (mg/cc)
1	New Fab antivenom	78.54
2	Purified hyperimmune monovalent equine plasma against cobra venom	60.84
3	Crude hyperimmune monovalent equine plasma against cobra venom	75.74

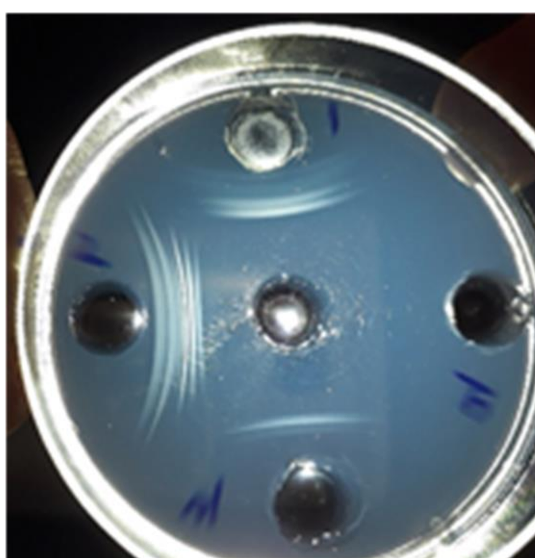


Figure 2. Ouchterlony test to demonstrate antigen and antibody reactivity. As displayed, the samples one (crude cobra plasma), two (hexavalent serum), and three (purified cobra plasma), due to having bivalent antibody molecules, show a visible precipitation band. However, sample four, which contains Fab fragment of monovalent antibody molecule, formed no visible band.

bands. However, well No. 4, which contained new Fab antivenom, formed no precipitation band, and this could indicate that the produced Fab antivenom contained monovalent Fab fragment and, therefore, formed no precipitation band.

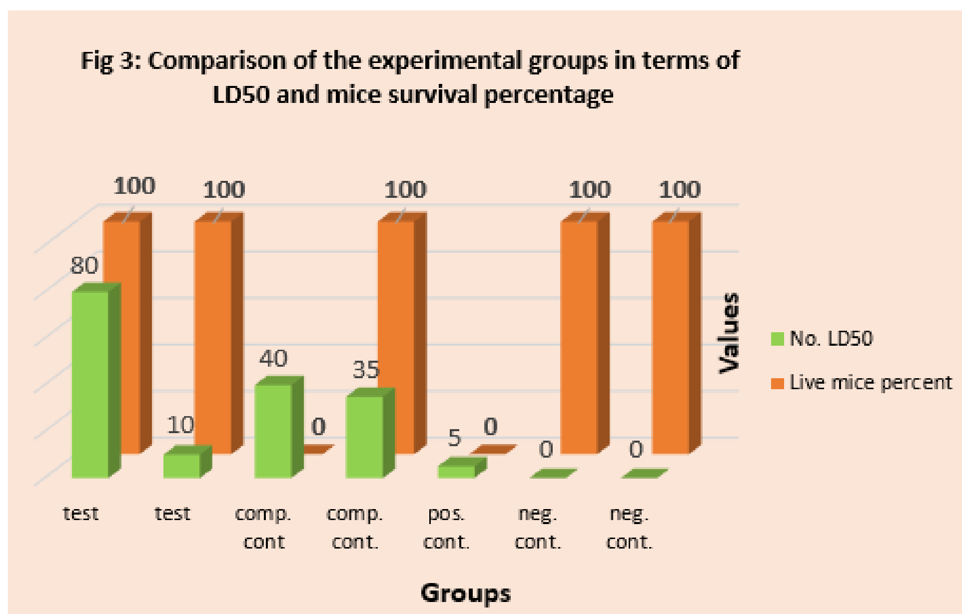
3.4. Potency test

The results of the potency test revealed that, the neutralization effect of Fab antivenom is higher than the primary monovalent anti-cobra venom plasma, so that each milliliter of the new Fab antivenom was able to neutralize a maximum of $80LD_{50}$ (624µg) of cobra snake venom, while the primary plasma was able to neutralize the cobra venom at a maximum of $35LD_{50}$ (273 µg) or, in other words, about 15% more efficient than the primary plasma in the same concentration (Figure 3).

4. Discussion

Venomous snakebites are serious threat, and annually around 5 million people worldwide get bitten by them, and the mortality rate is estimated to be more than 100,000 people. Most bites and fatalities occur in rural areas of tropical regions (7,13). The use of horse-derived antivenoms is the choice of treatment for snakebite victims, and they cause a rapid reduction in the symptoms of poisoning in the victims. However, the use of current antivenoms has some unwanted side effects, such as early anaphylactic shock and delayed serum sickness, which may be seen in some people treated by them. Before using horse antivenoms, many patients may have been sensitized to horse proteins, for example, through receiving anti-tetanus serum or in some people, they may have developed antibodies against these proteins (4).

Common antivenoms are usually divided into three groups based on their main and active component; Fab or $F(ab)_2$ fragment and intact IgG (25). Each of these antivenoms has specific advantages and weaknesses. For example, although the Fab fragment antibody with a molecular weight of about 50 kDa has faster tissue distribution and penetration and fewer side effects, its circulation duration in the body is less than the other two molecules (12). The antivenoms containing the $F(ab)_2$ fragment antibody with a molecular weight of about 100-110 kDa are intermediate between the other two, and current antivenoms are generally of this type. The third group, is less common, and its antibody component is usually intact IgG molecules with a molecular weight of about 150 kDa, and volume of its distribution in the body is slow, and side effects are more likely than the other two groups (4, 19). To produce Fab fragment antibody, enzymatic and chemical digestion or molecular and gene expression methods may be used, each of which has certain advantages and disadvantages (10, 26). For example, through molecular and gene expression in eukaryotic or prokaryotic cells, different amount of Fab fragment antibody, from 0.1 mg/liter to 1-2 g/liter of culture medium have been mentioned (15, 18, 19, 20, 22). In the enzymatic digestion method, it has shown efficiency from 10% to 35% (11, 15, 16, 26). The digestion efficiency obtained in this research, was 29.0%, which is a relatively good percentage compared to the works of other researchers. Various methods have been suggested for the purification of Fab and immunoglobulins, including ammonium sulfate (3, 21), caprylic acid (12, 17, 21), ion exchange, and L, A, and G proteins (11, 16). Some of these methods



Considering Figure (3) and Table 1, 35LD50 was the highest amount of cobra venom neutralized by primary hyperimmune plasma in the comparative control groups. While in the experimental groups, this number was 80LD50; therefore, similar to the negative control groups, all the mice survived.

are usually used together to increase their efficiency. In this research, the combined methods of ammonium sulfate and ion exchange were used, which according to the results obtained from SDS-PAGE and Ouchterlony tests, a large part of unwanted proteins were removed, while preserving the Fab fragment antibodies. For measuring neutralizing potency of antivenom, mice are usually used, during which, specific amounts of antivenom are mixed with venom whose lethality is determined in the form of LD_{50} and after a half-hour incubation at 37° C were injected to the mice. The criteria for deciding to increase or decrease the amount of venom and finally determining the strength of the antivenom, is the survival rate of the two-thirds of the mice within 24 h after the injection . In this research, NIH mice were used to determine the neutralizing potency of the produced Fab antivenom. The results of the potency test in mouse tail injection showed that each milliliter of produced Fab antivenom can neutralize 624µg ($80LD_{50}$) of cobra venom, which is more than the primary crude plasma with $35LD_{50}$ or 51 LD_{50} of the commercial hexavalent antivenom of Razi Institute respectively. According to the tests, 100% of the Fab antivenom injected mice survived. Based on the results of measuring the protein concentration of Fab antivenom and primary anti-cobra venom plasma with a Nanodrop device, the amount of Fb antivenom protein was 78.57 mg/cc and for plasma was 75/74 mg/cc, it can be seen that the protein concentration of Fab antivenom was only 0.04 times higher than the protein concentration of the original plasma, while its neutralizing potency has increased by 2.3 times outwardly, but in more precisely about 15% more efficient than the primary plasma in the same concentration. According to the results and specially the potency test, it seems that the produced new Fab antivenom can be proposed as a new product candidate alone or in combination with other antivenoms for treating snakebite victims.

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Authors' Contribution

Plasma Preparation: Dr zolfagharian and Mr zamen; Plasma purification: Mr saffar; Protein assay: Mr Hosseini; Potency test: Dr Mohammad pour and Mr Malekkhah; Administrative: Dr Motamed; Initial editing of the article: Dr Asli, Ms. Zand,

Ethics

It is declared that all ethical considerations were taken into account in the preparation of the submitted manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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