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Preparation and characterization of immunoglobulin yolk against the venom of *Naja naja atra*

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Chinese Cobra (*Naja naja atra*) bite is one of the leading causes of snake-bite mortality in China. The traditional anti-cobra venom serum therapy was found to be expensive and with high frequency of side effects. Therefore attempts were made to generate a high titer immunoglobulin from egg yolk (IgY) of crude cobra-venom immunized Leghorn hens, and to standardize an effective method for producing avian antivenom in relatively pure form. The IgY was isolated first by water dilution method to remove the lipid, then extracted by ammonium sulfate precipitation, and purified through anion exchange chromatogram. The different purities of IgY from different isolating stages were submitted to enzyme-linked immunosorbent assay and SDS-PAGE to determine their titers. Immunoblotting showed that the purified IgY (ion exchange chromatography fraction, IECF) recognized several antigenic fractions of cobra venom, and presented with the character of polyclonal antibody. IECF on SDS-PAGE under reducing conditions migrated as a 65 kDa heavy chain and a 35 kDa light chain, respectively. The LD_{50} of the *N. naja atra* venom was 0.62 mg/kg body weight in mice. Four times the LD_{50} dose of venom was selected as challenge dose, and the ED_{50} of IgY was 3.04 mg IECF/mg venom. The results indicate that the activity of anti-snake venom IgY could be obviously elevated by ion exchange chromatography, thus possessing therapeutic significance for snakebite envenomation.

Keywords: Egg yolk, Immunoglobulin, *Naja naja atra*, Snake venom

Snake bite is one of the most important causes of mortality in the world. In many parts of the world, especially in the tropical and subtropical countries of Southeast Asia, South America and Africa, snake bite is a serious medical, social and economic problem. Nearly 80% of the population of China live in rural areas, especially in the southern regions, and face the danger of snake bite. At present, although Chinese traditional herbal medicine are used popularly for treatment of snake bite, the antivenom is still the most effective method against snake bites in China. Since Dr Albert Calmette produced the first snake antiserum in donkeys against *N. kaouthia* venom in 1894, over 60 laboratories in more than 30 countries have developed over 80 separate antivenoms commercially[1,2]. In China, there are six antivenoms produced by Shanghai Institute of Biological Products, including *Deinagkistrodon acutus* antivenom, *Bungarus multicinctus* antivenom, *Agkistrodon halys* antivenom, *N. naja atra* antivenin, *Bungarus fasciatus* antivenom and *D. russelii siamensis* antivenom.

Antivenom is made by immunizing mammals such as horse, goat, rabbit with particular snake venom, and then the specific immunoglobulins are isolated from the blood. However, the production of antivenoms and their purification from mammalian blood have been found to be low yielding and labourious, and arousing many side effects in clinical therapy, including anaphylactic shock, pyrogen reaction, serum sickness, skin rash, laryngeal edema, low blood pressure, arthrosis ache, etc[2]. Due to recent advances made in technology of egg yolk immunoglobulins (IgY), the preparation of anti-snake venom IgY is a novel, safer, more convenient and inexpensive method for the manufacture of antivenom. There are several main advantages of IgY over the antibodies from mammals, including: (i) there is no need to bleed the animals; (ii) IgY does not cross-react with Fc receptors, heterophile antibodies and rheumatoid factor, and thus there is no risk of coming to falsely positive results in immunological assays; (iii) it is feasible to raise and immunize chickens; (iv) only a small amount of antigen is

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required. In 1996, the IgY-technology was recommended as an alternative method instead of mammalian IgG for supporting animal welfare by the European Centre for the Validation of Alternative Methods (ECVAM) workshop[3]. Several kinds of univalent IgY were produced from the egg yolks of immunized hens which could neutralize the venom of rattlesnake[4,5], *Bothrops* [6]. viper[7], indian cobra[8,9] and *Krait* [9]. In addition, a multivalent IgY against *Bitis* and *Naja* was prepared using *B. arietans, B. nasicornis, B. rhinoceros, N. melanoleuca,* and *N. mossambica* venoms to immunize chickens[10].

In the present study, the venom of *N. naja atra* has been used as antigen to immunize hens, and a modified method (including steps like water dilution, salting out and DEAE chromatogram) was used to remove lipid and isolate immunoglobulin from yolk. The purity, binding specificity and neutralization efficiency of isolated immunoglobulin have been examined.

Materials and Methods

N. naja atra venom—The snake *N. naja atra* was purchased from Chashanfu snake field (Guangzhou, China). The average length of the snake was 150 cm with an approximate age of 30 months. The snake venom was pooled by utensil biting during May to July, and then diluted by four volumes of the distilled water. The precipitates of crude venom were removed by centrifugation at 3000 *g* for 30 min, and lyophilized power (coded as NV-00406) was obtained by vacuum cryo- machine (FD-80L5, Japan) and kept in bottle at 4°C for use.

Breeding of hens—White 22-week-old Leghorn hens (16) weighing 1 kg each, obtained from Likang Poultry Farm, in good health and laying condition (laying 5-6 eggs per week) were used to produce IgY against venom (NV-00406). They were kept in individual cages with standard food and water, and all the animals were maintained according the Animal Welfare International Recommendations[11].

Lethality of venom—Venom (NV-00406) was intraperitoneally injected (0.5 ml in saline) into NIH mice. Mice of either sex weighing 18~20 g were divided into 6 groups of 10 each and received the doses (0.30, 0.37, 0.46, 0.58, 0.73 and 0.91mg/kg) intraperitoneally. The results were recorded 72 h after the ip injection of the venom into mice. The LD_{50} dose was calculated according to the method of Bliss[12] and expressed as μg per mouse.

Immunizations—Crude venom (75 μg) was resolved in 0.5 ml normal saline and mixed well with 0.5 ml of Freund's complete adjuvant (Sigma, San Raphael, USA) in a syringe to form an antigen emulsion which was injected into the muscle of each hen at chest, legs and wings for the initial immunization. Chickens received booster doses of 150μg crude venom with incomplete Freund's adjuvant (Sigma, San Raphael, USA) on 15 and 22 day after the first immune injection to raise the antivenom level in egg yolk. Eggs were collected daily one week after the first booster and the titer of specific antibody was determined in an aqueous extract of yolk by ELISA as described below. The eggs were stored at 4°C before use.

Isolation of IgY—Water dilution: The egg yolk was separated from the egg albumen, and then rolled on the filter-paper to remove the white proteins as completely as possible. The yolk membrane was punctured and the yolk contents were collected and mixed with nine volumes of distilled water. The *p*H of mixture was adjusted to 5.1 with acetic acid, and then stored at 4℃ for at least 6 h before centrifugation at 19,000 *g* for 15 min at 4°C. The lyophilized powder called as water dilution fraction (WDF) was prepared from half of the clear supernatant, and the other half supernatant was mixed with solid ammonium sulfate (AMS) for further purification.

AMS precipitation*:* AMS was added to the supernatant of water dilution till the saturation to 60% (390 g/L), and the mixture was stirred at 4°C for 2 h. The precipitate was collected by centrifugation and washed once with 60% saturated ammonium sulfate (SAS). The pellet was dissolved in PBS (*p*H 7.4), concentrated and desalted through ultra-filtration units (MWCO 100 kDa, Vivascience, Hanover, Germany), lyophilized as salting-out fraction (SOF).

Ion exchange chromatography—The immuno-globulin fraction was further purified by ion exchange chromatography. The gel of DEAE sepharose FF (Amersham Pharmacia Biotech) was washed with deionized water and soaked overnight in the sampling buffer before packed. The SOF (200 mg/10 ml) was loaded on a column of 2.5 cm×40 cm which was equilibrated with the binding buffer (0.075 mol/L PBS, *p*H 7.0), eluted with elution buffer (0.15 mol/L PBS, *p*H 7.0) in a flow rate of 0.5 ml/min at room temperature (23°～26°C). The elution was monitored at 280 nm with a HD-9704 UV spectrophotometer

(Jingke Equipment, Shanghai, China) for the presence of proteins. The protein concentration was quantiffed with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The purity and titer of IgY in difference peak solution were determined by ELISA and electrophoresis. The solution mainly containing the IgY was pooled and lyophilized as ion exchange chromatography fraction (IECF).

ELISA—Indirect ELISA was performed to determine the titer of three kinds of IgY samples, viz, water dilution, AMS precipitation and ion exchange chromatography respectively. Cobra Venom was diluted with 0.1 mol/L carbonate buffer (*p*H 9.6), 100 µl diluted venom （20 µg/ml）was coated on each well of the 96 well plate and incubated at 4°C overnight. After washing four times with PBS (0.05%) Tween-20 (PBS-T), blocked unspecific binding by incubation with 3% albumin-PBS, 200 µl/well at 37°C for 120 min. Wells were washed again four times with PBS-T. Then 100 µl of IgY solution (diluted as appropriate, with PBS containing 1% BSA) was added to each well. Non-specificity IgY solution were used as controls that extracted from eggs laid before the initial immunization. Incubation occurred at 37°C for 90 min. Each well was again washed with PBS-T six times; then 100 µl of 1:10000 diluted HRP-conjugated rabbit anti-chicken IgY (Upstate Biotechnology, NY, USA) was added to each well and incubated at 37°C for 60 min. Each well was washed with PBS-T six times again; and added 100 µl of freshly prepared substrate solution (3,3',5,5'-tetramethyl benzidine in 0.1 *M* citric acid, 0.2 *M* phosphate salt, 0.75% H_2O_2, *p*H 5.4). The reaction was allowed to develop for 15 min at 37°C and was stopped by the addition of 2 *M* H_2SO_4 (50 µl/well). The results were read at 450 nm with an ELISA reader (SunRise, Tecan, Austria). Absorbance of sample was at least 2.1 times higher than that of control. Reverse value of the maximal dilution fold of yolk was used as antibody titer.

Electrophoresis—The purity and sub-unit molecular weight of IgY in WDF, SOF and IECF were determined by SDS-PAGE using 10% resolving gel, and 4% stacking gel. For getting the subunit molecular weight of the immunoglobulin, SDS-PAGE was carried out after reduction of the protein with DTT. To further confirm the neutralizing capacity of IECF against the venom (NV-00406), the method of non-reducing SDS-PAGE was modified with 12% resolving gel and 4% stacking gel. Before the sample was loaded in Lanes 2～5, 100 µg venom was mixed previously with progressively increased IECF solution from 75～200 µg, incubated in 37°C for 30 min, mixed with the loading buffer, then the standard procedure of SDS-PAGE carried out. Gels were stained with Coomassie Blue R-250 and destained according to standard procedures. The molecular marker (P7702V, New England Biolabs, Boston, USA) was used to estimate the molecular weight of the protein.

Western blot—The venom was separated on SDS-PAGE gels and transferred to PVDF filters using the Trans-Blot semi-dry transfer cell (Bio-Rad, Richmond, USA). The transfer was monitored by prestained See Blue molecular weight marker (Invitrogen, Carlsbad, USA). The filters were blocked with 5% non-fat dried milk powder, 0.05% Tween-20 in TBS (TBS-T) for 1 h at room temperature. After washing with TBS-T, the membrane was incubated overnight with appropriately diluted IECF (1:1000) in TBS-T containing 5% bovine serum albumin (BSA). After washing thrice in TBS-T, the membrane was incubated for 1 h with rabbit anti-chicken IgY-HRP (1:5000) conjugated in TBS-T containing 5% BSA at room temperature. The filters were washed thrice in PBS-T, and then visualized by DAB buffer.

Neutralization efficiency of IgY—In order to evaluate the neutralizing effect of IgY, the challenge dose of Venom (NV-00406) was four times the LD_{50} dose. Neutralizing ability of IECF is expressed as effective dose 50% (ED_{50}), defined as mg of IECF/mg of venom at which half of the injected animals survived. IgY at different concentration were mixed with the challenge dose of venom respectively. The mixtures were incubated for 30 min at 37°C, and then 0.5 ml injected ip into groups of 10 mice for each dose. Control animals received the same amount of venom dissolved in the same volume of saline with negative IgY. Survival was determined 72 h later. The ED_{50} for IECF was calculated by probit analysis as mentioned above.

Results

Purification of IgY—IgY was detected out 10 days after the first immunization, and the titer of WDF increased rapidly by ELISA from second immunization （1.28×10^4） to third immunization （2.56×10^4. Eggs (146) 21~34 days after the first immunization were used for extracting IgY. The half of supernatant from water dilution was lyophilized

into 27.74 g WDF, and 0.38 g WDF obtained from each egg. It was about 122.8 mg/egg SOF fractionized by 60% ammonium sulfate. SOF (200 mg) was applied to DEAE chromatogram each time for IgY purification. After 2.5 h elution, two protein absorbance peaks, peak I (PI) and peak II (PII), were collected separately. The IgY was identified mainly in PII which was named as IECF. When the quantity of SOF loaded on a column of 2.5 cm×40 cm was 200-300 mg/10 ml, the titer of PI was not assessed by indirect ELISA in a protein's concentration (35 mg/ml). After being concentrated, desalted by ultra-filtration and lyophilized, over 50.3 mg/egg IECF could be obtained. The recovery rate of IECF was about to 41% from SOF (Table 1). The elution pattern of IgY from DEAE chromatogram is shown in Fig. 1.

IgY purity and reaction with antigen in SDS-PAGE—The purity of WDF, SOF and IECF were analyzed by SDS-PAGE under reducing conditions (Fig. 2). More than ten faint bands and at least five dense bands were stained in Lane 2 loaded with WDF. Two bands with 65 kDa (heavy chain) and 35 kDa (light chain) were obvious in Lane 3 loaded with IECF and Lane 4 loaded with SOF. Compared with Lane 3, the bands were determined for hybrid proteins with molecular weight (MW) range from 97 to 220 kDa in Lane 2, and from 36 to 56 kDa in Land 2 and 4. Thus, highly purified IgY could be obtained from egg yolk by a series of steps (water dilution, AMS precipitation and ion exchange chromatography).

The binding activity of IECF with native *N. naja atra* venom (NV-00406) appeared in SDS-PAGE under non-reducing conditions (Fig. 3). Following elevated concentration of IgY, the bands of A～F found in Lane 6 tended to fade in Lane 5 and almost disappeared in Lane 2～4. These results demonstrated

Table 1—The relationship between the recovery ratio of protein and the titer of IgY fraction

	WDF[1]	SOF[2]	IECF[3]
ELISA (×10[4]) (mean±SE)	3.41±0.61	40.90±7.77*	81.92±13.93**
Total content of protein in 73 eggs (g)	22.19	8.96	3.67
Recovery ratio of protein (%)	100	40.37	16.53

$P < 0.05$; *vs WDF; **vs SOF
[1]water dilution fraction
[2]salting-out fraction
[3]ion exchange chromatography fraction

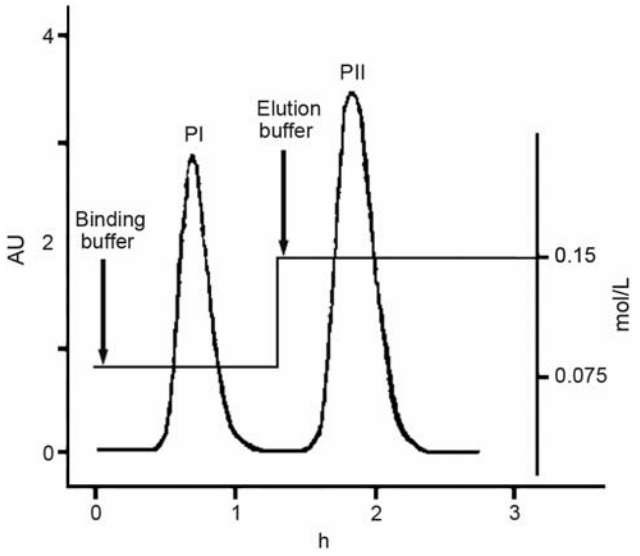

Fig. 1—IgY of salting-out fraction (SOF) further purified by ion exchange chromatography [The SOF (200 mg/10 ml) was loaded on a column of 2.5 cm×40 cm which was equilibrated with the binding buffer (0.075 mol/L PBS, *p*H 7.0), eluted with elution buffer (0.15 mol/L PBS *p*H7.0) in a flow rate of 0.5 ml/min at room temperature (23°～26°C). The IgY was identified mainly in PII]

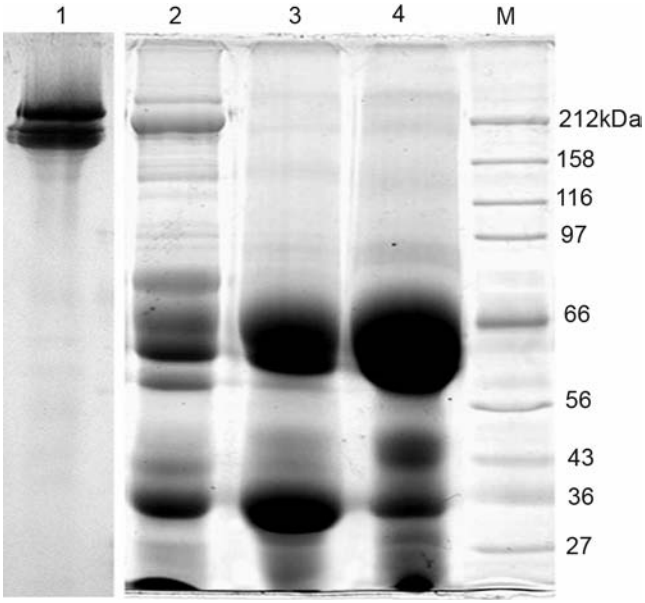

Fig. 2—Purities of WDF, SOF and IECF analyzed by SDS-PAGE [The purities of IgY and molecular weight of IgY sub-unit in WDF, SOF and IECF were determined by Coomassie Blue-stained SDS-PAGE using 10% resolving gel, and 4% stacking gel. The molecular weight of IECF was about 180-220 kDa which was shown in Lane 1 loaded with 50 μg protein under non-reducing conditions. Two bands with 65 kDa (heavy chain) and 35 kDa (light chain) were observed in Lane 2-4 loaded with 100 μg protein under reducing conditions. Lane 1, IECF; Lane 2, WDF; Lane 3, IECF; Lane 4, SOF; Lane M, Molecular weight standards]

that the compounds of antigen-antibody by preincubation are more difficulty to permeate through the 12% stacking gels than antigen itself.

Protein content and titer of IgY production—Half of the supernatant from water dilution was lyophilized into 27.74 g WDF including 22.19 g protein, the other half was reduced to 8.96 g SOF by following purification treatment with ammonium sulfate, then to 3.67 g IECF after the further ion exchange chromatography. The protein content of WDF, SOF and IECF assessed by BCA method was about to 80, 100 and 100% respectively. The recovery of the protein was calculated after the purification step (Table 1). The titers of three kinds of IgY production were assessed by indirect ELISA in the same protein concentration (35 mg/ml). The titer of IECF (81.92×10^4) was two fold higher than SOF (40.96×10^4), and 23 times higher than WDF (3.41×10^4). These results demonstrated that the titer of IgY increased dramatically along with the decrease of hybrid protein (Fig. 4).

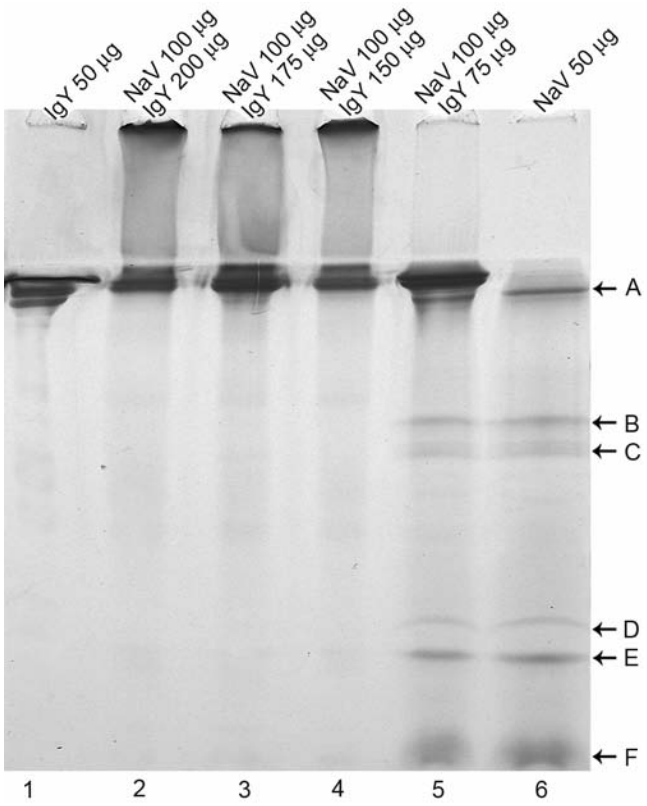

Fig. 3—Binding activity of IECF with *N. naja atra* venom appearing in SDS-PAGE under non-reducing conditions [The samples were analyzed by Coomassie Blue-stained SDS-PAGE in 12% resolving gel and 4 %% stacking gel. The bands (A~F) of *N. naja atra* venom (NaV) were obvious in Lane 6, tended to fade in Lane 5 and almost disappeared in Lane 2~4 with elevated concentrations of IgY from Lane 5 to Lane 2. Lane 1, 50 µg IECF; Lane 2, 100 µg NaV+200 µg IECF; Lane 3, 100 µg NaV+175 µg IECF; Lane 4, 100 µg NaV+150 µg IECF; Lane 5, 100 µg NaV+75 µg IECF; Lane 6, 50 µg NaV]

Western blot—The venom (NV-00406) was a complex mixture of antigens. At least 14 protein bands, with the molecular weight ranging from 8 kDa to over 100 kDa, were visible in Lane 1 by SDS-PAGE which were stained with Coomassie Blue R-250. After being transferred to PVDF filters and visualized by DAB buffer, several major binding bands were shown in Lane 2~4 with the molecular weights of 8~20, 50~55, 65 and 100 kDa. Compared with Lane 1, DAB-stained bands on Lane 2~4 corresponded to those high-abundance ones which were stained by Coomassie Blue (Fig. 5). It still remains unclear whether some antigens of low-abundance were lost during the transfer to PVDF filters, or some IgY-subunits were insufficient in binding activity.

Lethality of venom and neutralization efficiency of IgY—The LD_{50} of venom was 0.62 mg/kg body weight of mice. All mice were killed within 2 h after injection of 4 LD_{50} (2.48 mg/kg, defined as challenge dose) of venom. Various dilutions of IgY (4~9 mg/kg) were mixed with the challenge dose of venom, and the neutralizing ability of IECF was obvious in dose-response relationship. The ED_{50} of IECF was 3.04 mg of IECF/mg of venom. When IECF/venom ratios rose

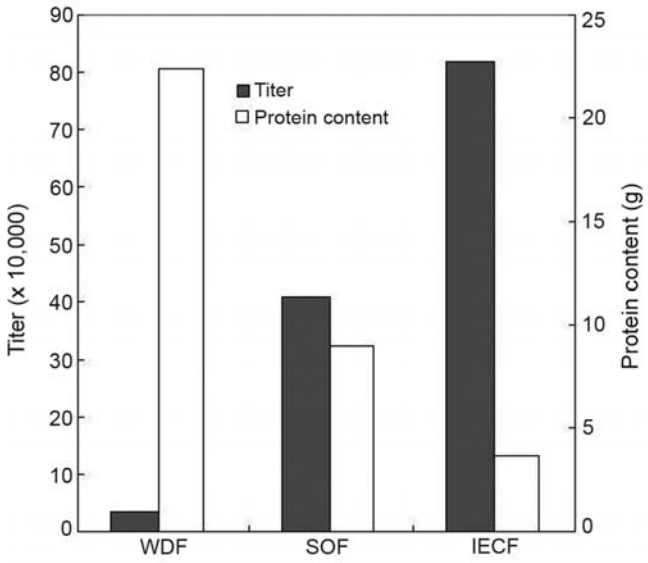

Fig. 4—The relationship between the total protein contents and the titers of three kinds of IgY(WDF, SOF and IECF) [The white-strip represents the protein content (WDF 22.19 g, SOF 8.96 g and IECF 3.67 g), and the grey-strip contributes to the titer (WDF, SOF and IECF)]

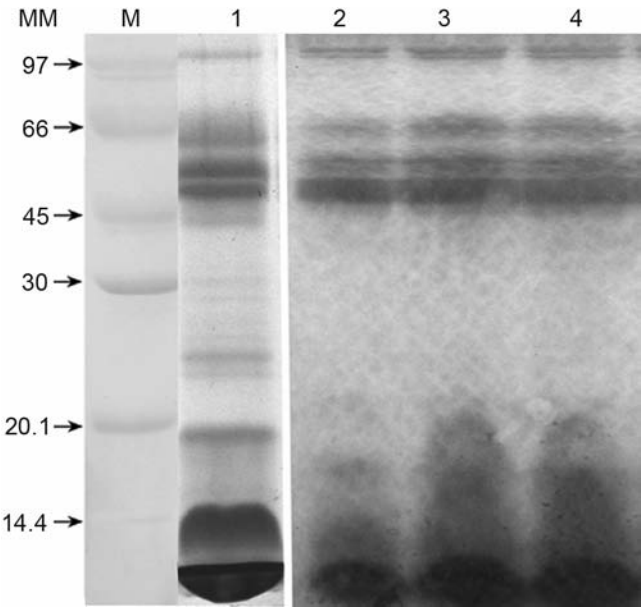

Fig. 5—The Characterization of polyclonal IgY as analyzed by Western blot [Lane M, protein standards; *N. naja atra* venom was separated by SDS-PAGE in Lane 1 (100 μg venom loaded), stained with Coomassie Blue R-250, and Lane 2 (80 μg), 3 (100 μg) and 4 (120 μg) were treated with Western blot, showing several DAB-stained bands with the molecular weight of 8~20 kDa, 50~55 kDa, 65 kDa and 100 kDa]

to 3.62 (9.0 mg/2.48 mg), all animals were survivors without obvious toxic reactions (dyspneic respiration, myasthenia of limbs, tetraplegia and paralysis of respiratory muscle).

Discussion

By proteomic analysis 124 categories of proteins and peptides were identified in venom of *N. naja atra*[13], which may be divided into two groups according to their molecular mass and functions: (i) the spreading factors that generally raise the diffusion of toxic components in organism, including Cobra venom factor (137 kDa)[14], phosphodiesterase (110 kDa), hyaluronidase (69 kDa)[15], and natrahagin (a hemorrhagic matrix metalloprotease, 47.1 kDa)[16] and so on; (ii) three major toxic components, including short-chain (60~62 amino acids) and long-chain (70~73 amino acids) neurotoxins[17], cytotoxins (CTX I-V and n, 6-7 kDa)[18, 19] and phospholipase A_2 (PLA_2, 13.2 kDa)[20,21]. The venom can cause fatal physiological disorders, e.g. neurotoxicity, haemolysis, lysis of cell membrane, as well as cardiac and muscular failures[22,23]. The present data showed that the LD_{50} of *N. naja atra* venom (NV-00406) in mice was assessed as 0.62 mg/kg, and all animals would have died within 2 h after injection with the challenge dose (4 LD_{50}) of venom if specific IgY was not administered in time,.

In recent years, chickens are recognized as a convenient and inexpensive source of antibodies. The purification methods for IgY generally can be divided into three principal steps: water dilution, involving the removal of lipids from the diluted egg yolk[24], salt precipitation and chromatographic methods, involving isolation of immunoglobulin from egg yolk protein[25,26]. A two-step method for isolation of antiviper venom antibodies from egg yolk has also been reported[7], which including the first step of water dilution followed by the second step gel filtration yielding > 90% pure IgY. The reported isolation and purification methods of IgY from egg yolk were modified and obtained more pure IgY. A three step method was set up in the present experiment for removal of lipids from the diluted egg yolk by water dilution, elimination of greasy impurities and part of proteins by solid ammonium sulfate, and further IgY purification by ion exchange chromatography. Approximately 50.3 mg/egg of pure IgY could be obtained by the three step method and a good antigen binding capacity was proved. The present results from Western blots clearly showed that different IgY subunits from ion exchange chromatography specifically binded to their corresponding antigens with MW ranging from larger than 100 kDa to lower than 10 kDa. Therefore, a specific polyclonal IgY was produced in the present study against different components of native venom through immunization of hens.

N. naja atra venom could be divided into more than 12 bands with MW ranging from 8 kDa to over 100 kDa by SDS-PAGE (Fig 5). After preincubation of IgY with the venom, the band A~F of venom in the electrophoretic pattern were blocked in the top of lane (Fig. 3). This disappearance and fading of bands may be related to the antigens bound completely by IgY, forming immune complexes that were more difficult to permeate through the gels than antigen or IgY alone. This result is different from the change in the SDS-PAGE pattern of *B. asper* venom induced by protein hydrolysis[27]. Similar to Western Blot, the capability of IgY in antigen recognization and conjugation can also be shown by SDS-PAGE, but SDS-PAGE is more convenient and cheap in experimental performance.

Neutralization or protection studies are common methods *in vivo* to assess the characteristic of IgY in

specificity and activity. A fixed dose of antivenom preincubated with various dilutions of venom was assessed in some earlier neutralization studies, and neutralizing capacity of antivenom against venom was expressed as the number of LD_{50} doses neutralized by 1 ml of serum[28,29]. Then, a fixed dose of monoclonal antivenom preincubated with a fixed dose of cobrotoxin or neurotoxin was used to observe the increase for the time to death compared to control animals, and neutralizing ability of antivenom against the venom was defined as a component /antibody molar ratio[30, 31]. In recent years, many neutralization studies have been performed by using a fixed dose of venom preincubated with various dilutions of equine antivenom. A fixed dose of snake venom, i.e., challenge dose, was selected at 3, 4 or 5 LD_{50}[32-34] to absolutely kill all animals in short times. Neutralizing ability of antivenom against the challenge dose is expressed as ED_{50}, defined as the antivenom/venom ratio in which the activity of venom is reduced to 50% when compared to the effect induced by venom alone.

Though venom and antivenom injected independently was considered to simulate the real conditions of an envenomation[35], preincubation of venom and antivenom is used more extensively in neutralization experiments, since results do not depend on pharmacokinetics of venom and antivenom, but on concentration and neutralizing ability of antibodies presented in the antivenom. In a standardizing preincubation protocol, antivenom/venom was incubated together for 30 min at 37°C[31], then the mixtures were injected ip into mice.

In the present neutralization experiments, four LD_{50} challenge doses of the venom were selected and IgY/venom complexes injected directely into mice, following the increased ratio of IgY/venom from 3.04 (ED_{50}) to 3.62, the mortality of mice was reduced from 50 to 0%. If immune complex was removed through centrifugation and the supernatant was injected only after incubation, the effective neutralization ratio of IgY to venom would close to the results showed on SDS-PAGE. Therefore, in the present study a high titer of anti-cobra venom antibody was produced through immunizing hens with cobra venom, which may lay a foundation for therapy of cobra bite and for generation of other anti-venom antibody.

Conflict of interest—The authors declare no conflicts of interest.

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