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Bioinformatics-based design of novel antigenic B-cell linear epitopes of *Deinagkistrodon acutus* venom

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Abstract. – OBJECTIVE: With the help of bioinformatics analysis, we wished to develop a novel antivenom against the *Deinagkistrodon (D.) acutus* snake venom using B-cell linear epitopes of three primary toxins (serine protease, metalloprotease, and phospholipase A2).

MATERIALS AND METHODS: cDNA sequences of three toxins of *D. acutus* venom were retrieved from the NCBI database. B-cell linear epitopes were predicted using DNAStar and the website server software provided by IEDB. Then, the sequences of the predicted epitopes were artificially synthesized and inserted into the vector pET-32a-c(+). Recombinant antigen peptide was expressed and purified. BALB/c mice were immunized with the recombinant antigen peptide. The immunoprotective effect of this novel antivenom was measured by neutralization of venom haemorrhagic activity.

RESULTS: Six epitopes were obtained by bioinformatics analysis. ELISA analysis showed that antibody titre was >8,000 against snake venom and >64,000 against the recombinant peptide. Neutralization assays confirmed that the developed antivenom could effectively reduce the haemorrhagic activity of snake venom.

CONCLUSIONS: Six B-cell linear epitopes of *D. acutus* snake venom were predicted by bioinformatics analysis and successfully utilized to produce a novel antivenom.

Key words:
Deinagkistrodon acutus, Snake venoms, B-cell linear epitopes, Bioinformatics.

Introduction

Venomous snake bites are a relatively common occurrence in tropical and subtropical regions. Without proper and timely treatment, they often cause death or disability[1,2]. At least 2.5 million venomous snake bites occur every year worldwide, resulting in 125,000 deaths[3]. A critical role in the treatment of snake bites is attributed to antivenom. It is a specific antidote, which, however, may cause severe adverse reactions such as anaphylactic shock, urticaria, hypotension, asthma, and thrombotic phlebitis. The prevalence of these adverse reactions can reach up to 20%[4]. Another limitation of antivenom is that it contains antibodies to non-toxic components, which reduces its therapeutic effect[5]. Therefore, improving the efficacy and safety is one of the main objectives of novel antivenom development.

With the advent of bioinformatics and immunoinformatics, a new approach to identifying protein epitopes based on computer simulation and bioinformatics has emerged. Recent examples of such approach are presented in the reports by Wagstaff et al[6] and Machado de Avil et al[7]. The former report predicted seven epitopes of snake venom metalloprotease (MP) from carpet viper and found that the serum produced by immunized animals can effectively neutralize venom-induced haemorrhaging. The latter report predicted five epitopes of snake venom MP consisting of 12 amino acids and demonstrated the corresponding antivenom to exhibit good protective effects.

In the present study, we analyzed cDNA sequences of three major toxic components (serine protease (SP), MP, and phospholipase A2 (PLA2)) of *Deinagkistrodon (D.) acutus* venom using bioinformatics methods. *D. acutus* is a venomous snake distributed in southern China and in some areas of Vietnam[8]. In addition, we predicted key antigenic epitopes of three snake venom toxins and prepared the corresponding antivenom using tandem expression of predicted epitopes. The protective effect of this antivenom was then successfully demonstrated in an animal study.

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Materials and Methods

Mice

BALB/c mice weighing 18-20 g were provided by the Animal Center of the Third Military Medical University (Chongqing, China). All animal experiments were performed in strict accordance with the regulations of the Guide for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of the People's Republic of China. All efforts were undertaken to minimize animals' suffering. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

Sequence alignment of snake venom proteins

Six cDNA sequences were selected from the NCBI database (http://www.ncbi.nlm.nih.gov) for each of the three *D. acutus* snake venom toxins. The accession numbers of the sequences were MP – AJ223283, AY566610, AF117637, AJ223283, GU290061, and GQ245980; SP – AF159060, AJ006471, AY861382, EF093559, EU681180, and EF101918; and PLA2 – AJ223188, X77648, X77649, AF269132, X77650, and X77651. The selected sequences were aligned in the MegAlign module of the DNAStar software[9] according to the type of the toxins. One sequence of each toxin that exhibited the highest homology with the common sequence was chosen for epitope prediction.

Linear epitope prediction of snake venom protein

Linear epitope prediction of snake venom protein sequences was performed using the IEDB online software (http://tools.immuneepitope.org /tools/bcell/iedb_input) with the following indicators: Chou and Fasman β turn, Emini surface accessibility, Karplus and Schulz flexibility, Kolaskar and Tongaonkar antigenicity, and Parker hydrophilicity[10]. Secondary structure prediction of snake venom proteins was performed using the Protean module of DNAStar with the following antigen prediction indicators: protein sequence flexibility, surface accessibility, hydrophilicity, and Jameson-Wolf antigenic index[11,12]. Based on the prediction results of the two software packages, the regions with good consistency were identified to determine the epitopes of each venom protein sequence. According to the literature[7], two epitopes, consisting of 12 amino acids each, were selected for each venom toxin.

Tandem expression of predicted epitopes and preparation of the antivenom

All six linear epitopes predicted by the software were aligned together using the software, with each antigen fragment being separated by lysine and serine[13]. After optimization of the nucleic acid codon, the gene sequence design of the antigenic epitope peptide was sent for sequence synthesis, epitope peptide expression, and purification. BALB/c mice were immunized with the prepared epitope peptide used as an antigen (50 μg per injection per mouse). For first immunization, the epitope peptide was completely emulsified with an equal volume of complete Freund adjuvant and administered by subcutaneous injection into the bilateral axilla and digastric groove. For second and third immunizations, the antigen was mixed, emulsified with incomplete Freund adjuvant, and administered with a two-week interval. Mouse blood specimens were collected one week after the third immunization. The antibody titre was assessed by indirect ELISA as described below.

Antibody titre determination by indirect ELISA

Blood specimens were collected five weeks after animal immunization with the recombinant antigen peptide. Serum specimens were obtained. The antibody titre against snake venom or recombinant antigen peptide was determined by an ELISA using the following steps. First, the specimens were diluted to 0.2 μg/mL (to determine the antibody titre against snake venom) or 0.5 μg/mL (to determine the titre against recombinant antigen peptide) with an antigen-coating buffer (0.1 M carbonate, pH 9.6). Diluted specimens (100 μL per well) were pipetted onto 96-well plate. The plate was incubated overnight at 4° C and washed with a washing buffer (PBS pH 7.4, 0.05% Tween-20). Then, 150 μL of blocking buffer (PBS pH 7.4, 0.05% Tween-20, 2% BSA) was added to each well. The plate was incubated for one hour at 37° C and then washed with the washing solution. Immunized mouse serum was diluted to an appropriate concentration with an antibody dilution buffer (PBS pH 7.4, 0.05% Tween-20, 1% BSA). Diluted serum (100 μL per well) was added to 96-well plate along with immunized mouse serum as negative control and antibody dilution buffer as blank control. After 30 min incubation at 37° C, the plate was washed, and 100 μL of TMB substrate solution were added to each well. The calorimetric reac-

tion was allowed to proceed for 15 min in the dark, after which the reaction was terminated by addition of 50 μL of stop solution (2 mol/L H_2SO_4). Optical densities were determined at a wavelength of 450 nm using a microplate reader.

Identification of immunoprotective antigen by neutralization of haemorrhagic activity

Neutralization of haemorrhagic activity was performed according to a previously published procedure[14] with some minor modifications. Dilutions of snake venom were prepared in PBS and intradermally injected into the back skin of BALB/c mice (0.1 mL/injection). The mice were sacrificed 24 hour later, and back skin was removed to measure the diameter of subcutaneous haemorrhagic spots. The minimum hemorrhagic dose (MHD) was defined as the dose of snake venom that produced a haemorrhagic spot of 10 mm in diameter. The MHD of *D. acutus* venom was estimated to be 4 μg. In experimental group, 50 μL of the antivenom prepared from the recombinant antigen peptide were mixed with either 2 or 4 MHD of the snake venom solution in a final volume of 100 μL. The mixture was incubated for 30 min at 37° C and intradermally injected into the back skin of BALB/c mice. Mice in a positive control group was treated with 2 or 4 MHD of the snake venom (100 μL venom solution), whereas mice in a negative control group received injection of 100 μL PBS. Each animal group comprised four animals. The mice were sacrificed 24 hour later. The diameter of subcutaneous haemorrhagic spots was measured, and haemorrhagic area was calculated.

Results

Sequence alignment of snake venom proteins

cDNA sequences of three selected venom toxins were aligned using the MegAlign module of DNAStar. MP GU290061 (Figure 1A), SP AF159060 (Figure 1B), and PLA2 AJ223188 (Figure 1C) showed the highest homology with the common sequence. Therefore, the above three sequences were selected for secondary structure analysis and antigenic epitope prediction.

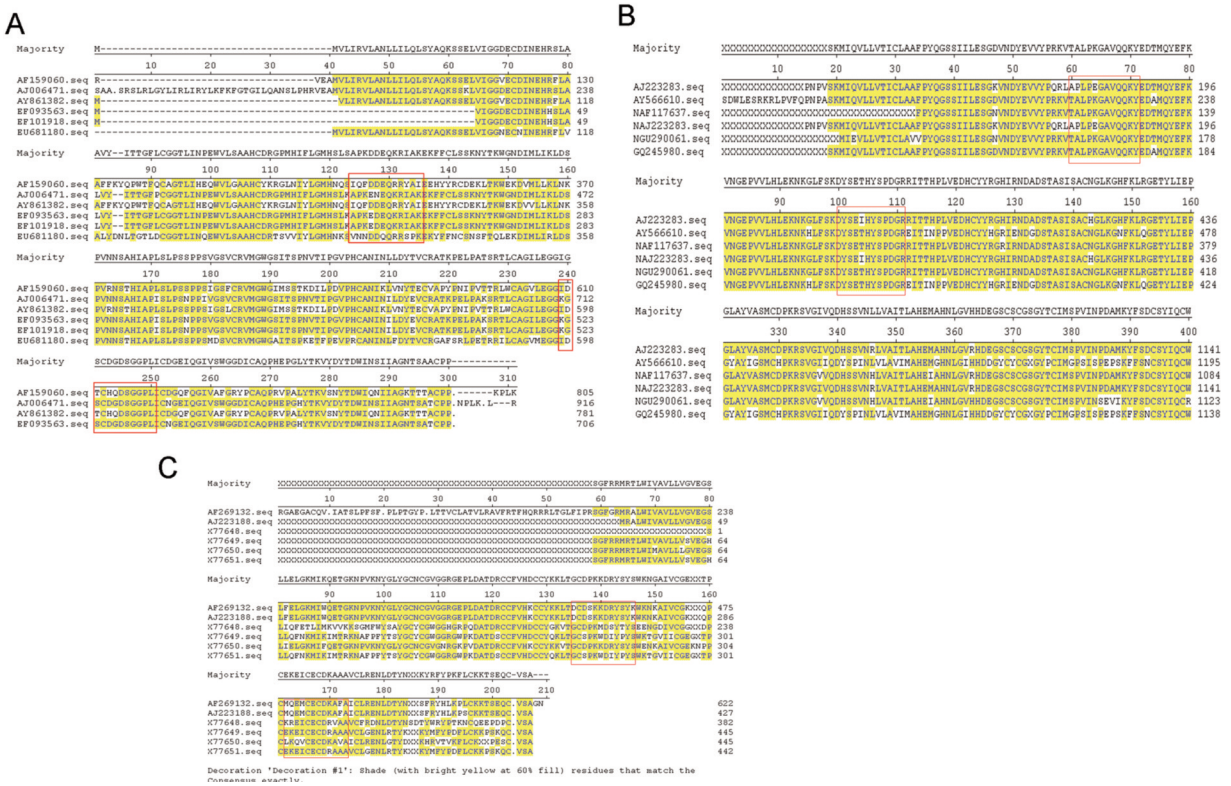

Figure 1. Comparison of sequences of three major toxins of the venom of *D. acutus*. **A**, Serine protease. **B**, Phospholipase A2. **C**, Metalloprotease. The fragment marked with a red square is the position of the epitope in the sequence comparison.

Epitope prediction of snake venom proteins

Secondary structure analysis of SP AF159060 was performed using the Protean module of DNAStar. The hydrophilicity, hydrophobicity, Jameson-Wolf antigenic index, and surface accessibility of venom proteins were comprehensively analyzed using DNAStar. Furthermore, the Emini surface accessibility, Karplus and Schulz flexibility, Kolaskar and Tongaonkar antigenicity, and Bepipred linear epitope prediction were analyzed using the IEDB online software for linear epitope prediction of proteins. The epitopes obtained by the software were comprehensively analyzed. Different prediction methods commonly revealed suitable epitope features in the segments 82-105 and 196-218. Two peptides consisting of 12 amino acids were selected from the above two segments as the predicted epitopes: SE1 (IQFDDEQRRYAI) and SE2 (IDTCNQDSGGPL). The results of sequence alignment showed that these two peptides were highly conserved. Epitope prediction of MP GU290061 and PLA2 AJ223188 was performed following the procedure described previously, and two 12 amino acid long epitopes were selected from the sequence of each snake venom protein: MET1 (TALPKGAVQQKY) and MET2 (DYSETHYSPDGR), and PL1 (MQEMCECDKAFA) and Pl2 (DCDSKKDRYSYK). In total, six epitopes were obtained (Figure 1).

Recombinant tandem expression of predicted epitopes

The six predicted epitopes were combined, separated by lysine and serine. This was followed by codon optimization and whole gene synthesis (Figure 2). The obtained DNA sequence was inserted into the plasmid pET-32a-c(+) at the restriction sites *Kpn*I/*Xho*I. The expressed and purified recombinant antigen peptide was analyzed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3). The molecular weight of the recombinant antigen peptide was estimated to be about 35 kDa. The protein band was relatively wide; this was considered to indicate partial degradation of the recombinant peptide.

Figure 2. Recombinant antigen peptide sequences of DNA. DNA sequences for six epitopes are marked with red ink. DNA sequences marked with black ink indicate connections between two epitopes.

Antibody titre

Antibody titre was determined by indirect ELISA using the serum of mice immunized with the recombinant peptide (Figure 4). The antibody titres of immunized mouse serum were >8,000 against snake venom and >64,000 against the recombinant peptide. These results indicated that the recombinant antigen peptide showed better immunogenicity.

Neutralization of haemorrhagic activity

No haemorrhagic spot was observed on the skin of mice treated with PBS. As showed in Figure 5, the areas of haemorrhagic spots were significantly reduced and had a lighter colour in experimental mice treated with the antivenom prepared from the recombinant antigen peptide. At the venom dose of 2 MHD, the antivenom reduced the haemorrhagic area by 81%, and this reduction reached 92% with the venom dose of 4 MHD. The results indicated that the recombinant antigen peptide showed better immunoprotective activity.

Discussion

Snake venom from *D. acutus* has a complex composition and contains many proteins, carbohydrates, lipids, and bioactive peptides. Several of these compounds act as blood poison, and can

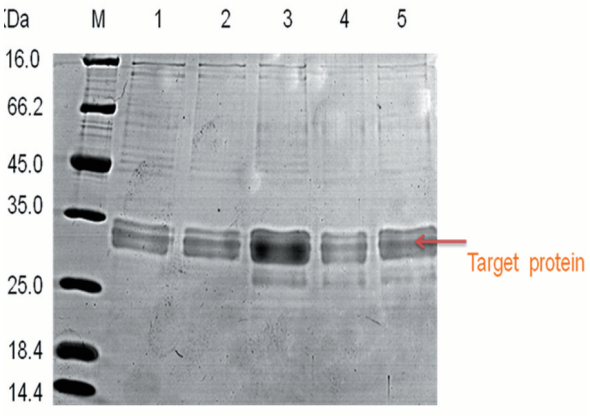

Figure 3. Electrophoretic separation of recombinant antigen peptide on 10% SDS-PAGE. M: Marker. 1-2: Urea + 20 mM Imidazole (5 resin volumes). 3: Urea + 50 mM Imidazole (5 resin volumes). 4: Urea + 500 mM Imidazole (5 resin volumes); sample volume 5 µl. 5: Urea + 500 mM Imidazole (5 resin volumes); sample volume 5 µl.

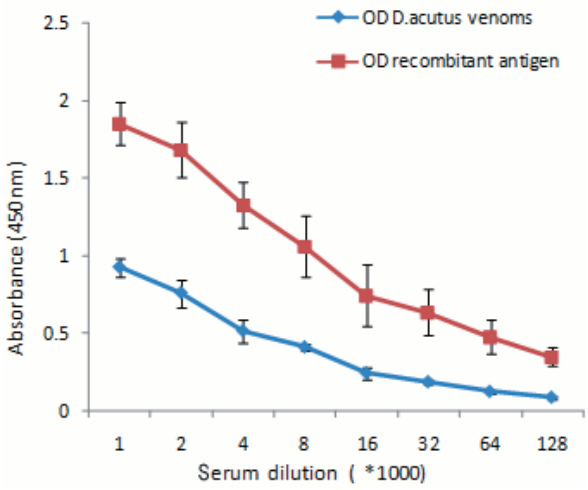

Figure 4. ELISA antibody titre. Antibody titres in mice immunized with D. acutus venom or recombinant antigen peptide.

cause extensive hemorrhaging and local tissue necrosis. The major toxin components identified in the snake venom include MP, SP, and PLA2. The snake venom MP belongs to the zinc MP family and is the primary active component of venom proteins. MP can be divided into four classes according to their protein structure: class PI, which contains only the MP domain, class PII, which contains MP and disintegrin domains, class PIII (MP, disintegrin, and cysteine-rich domains), and class PIV (a disulfide-linked C-type lectin domain in addition to the domains present in class PIII MP)[15]. The main physiological function of MP is to destroy the blood coagulation system of the prey, leading to systemic haemorrhaging. Moreover, MP can induce apoptosis and inflammatory reactions. The snake venom SP belongs to tryptase subfamily and mainly affects components of the coagulation cascade, as well as fibrinolytic and kallikrein-kinin systems, thereby causing an imbalance of the coagulation system of the prey[16]. The snake venom PLA2 catalyzes the hydrolysis of phosphate triglycerides to produce fatty acids and lysophospholipids. The toxic effects of PLA2 mainly include anticoagulation, hemolysis, muscle toxicity, cytotoxicity, neurotoxicity, and edema. PLA2 is often called myotoxin and neurotoxin[17]. The synergy of the three snake venom toxins leads to systemic haemorrhaging and tissue necrosis in patients.

Antigenic epitopes are special structures and immunoactive groups on the surface of an antigen molecule. Epitopes contain a site that can cause antibody production. These antibodies recognize the epitope[18]. Based on their structure, epitopes can be divided into linear and conformational types. Presently, epitope prediction by bioinformatics analysis mainly focuses on B-cell linear epitopes. In contrast, conformational epitopes are highly complex, resulting in increased prediction difficulty and low accuracy[19]. When predicting epitopes by bioinformatics analysis, the hydrophilicity, surface accessibility, plasticity, and turn structures of the selected

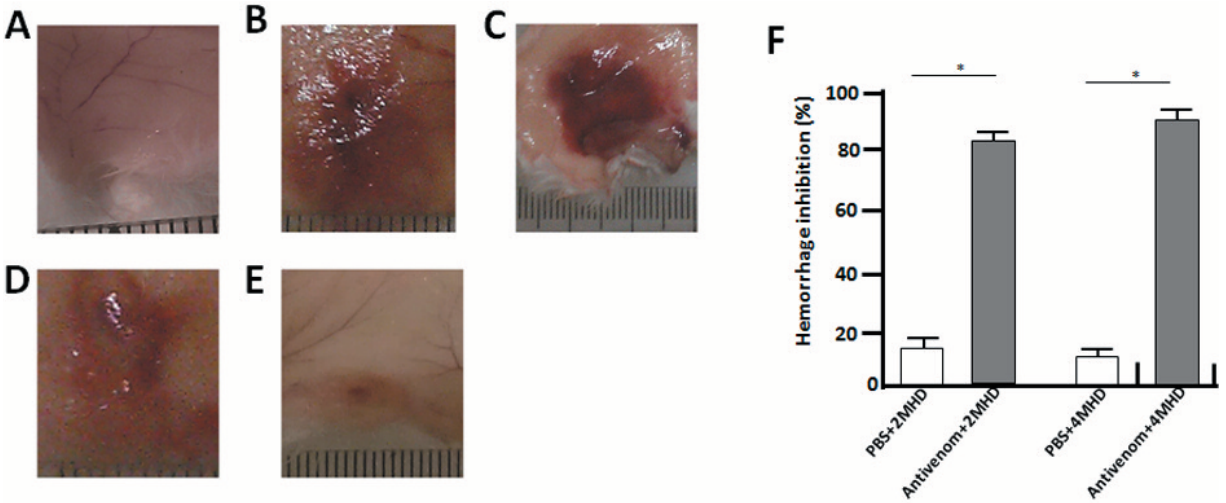

Figure 5. Neutralization of haemorrhagic effect of D. acutus venom in vivo. Haemorrhage was assessed on the visceral side of the mice skin samples taken 24 hours after exposure to **D**. acutus venom. **A**, Haemorrhage induced by PBS served as negative control. **B**, Hemorrhage induced by 2 MHD of **D**. acutus venom. **C**, Haemorrhage induced by 4 MHD of **D**. acutus venom. **D**, Haemorrhage induced by 2 MHD of **D**. acutus venom plus antitoxic sera. **E**, Haemorrhage induced by 4 MHD of **D**. acutus venom plus antitoxic sera. **F**, Haemorrhage inhibition (%) in the presence of antitoxic sera.

segments need to be taken into consideration. Furthermore, production of appropriate sequences consisting of 12-15 amino acids is favourized[20]. Newer technologies, such as artificial neural networks and support vector machine algorithms, permit the use of protein tertiary structure in linear epitope prediction, development of B-cell epitope analysis software (e.g., Bcipep, ABCpred, BepiPred, APP, CEP, DiscoTope, ClusPro, PPI-Pred, and IEDB), and establishment of databases[21]. Therefore, B-cell epitope prediction using bioinformatics technology is an important method used to determine new B-cell epitopes. Not surprisingly, it gained wide popularity.

Previous prediction and screening methods of snake venom antigens mostly focused on a single toxin component[22] and antibody preparation against this selected toxin. However, since snake venom has a complex composition and since venom's multiple toxins often synergise, in the present study we selected the three most important toxin components of snake venom from *D. acutus*: MP, SP, and PLA2. Selected protein sequences were used to predict B-cell linear epitopes in order for the antivenom to recognize and neutralize all three components of the snake venom to achieve a better protective effect.

We took advantage of two software tools for epitope prediction to comprehensively analyze the antigen fragment in terms of hydrophilicity, surface accessibility, flexibility, the Jameson-Wolf antigenic index, and BepiPred linear epitope prediction. The results were mutually confirmed by the selection of fragments with good consistency as the predicted epitopes, thus ensuring the accuracy of prediction.

Snake venom from *D. acutus* is mainly a blood poison, and its toxicity is primarily manifested as haemorrhagic activity. Therefore, we selected neutralization of haemorrhagic activity as the indicator used to evaluate the protective effect of the antivenom. In the animal experiment, the area of the haemorrhagic spot on the skin of the immunized mice was markedly reduced and showed a lighter colour. These results indicate that the antivenom designed using the predicted epitopes effectively neutralizes the venom-induced haemorrhaging.

Conclusion

Our study successfully defined six epitopes of three major toxin components of *D. acutus* venom by bioinformatics analysis. Based on tandem expression of the six epitopes, the antivenom obtained from immunized mice could effectively neutralize venom-induced haemorrhaging. This provides foundation for development of new antivenoms.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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