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Immunoaffinity chromatography in antivenomics studies: Various parameters that can affect the results

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ABSTRACT

Antivenomics is a recently developed powerful method for the study of antivenom antibody profiles when bound to homologous and heterologous snake venoms. The information obtained is useful in gaining an understanding of venom protein immunogenicity, antivenom potency and also for the improvement of antivenom potency and paraspecificity. The preferred method used in this type of study is immunoaffinity chromatography of the venom proteins on an antivenom IgG (or $F(ab')_2$) column where the bound and unbound proteins can be separated and identified. However, there are some parameters of the immunochromatography that can significantly affect the binding of the proteins to the immunoaffinity matrix and lead to imprecise results in antivenom immunoprofiling. The present study demonstrated that the ligand density (mg IgG/ml of the matrix), the buffers used for binding and washing the venom proteins, the amount of venom loaded, the abundance of some venom protein(s) and the eluting buffers can significantly alter the binding of the proteins to the matrix and consequently the conclusions drawn from antivenomics studies. Furthermore, the immunochromatographic procedure can be extended to include the estimation of the relative affinity of venom protein-antibody interactions that can provide additional information useful to antivenomics study.

1. Introduction

Antivenomics is a recently developed method to determine whether antibodies in an antiserum/antivenom can bind to specific venom proteins (Calvete et al., 2009; Gutierrez et al., 2008; Lomonte et al., 2008). For a homologous venom-antivenom pair, the results can yield information relating to the immunogenicity of individual venom protein(s). For a heterologous venom/antivenom pair, the information obtained can demonstrate the cross reactivity of antivenom antibodies towards any of the venom proteins/toxins. The information obtained from antivenomics studies can be very useful in understanding antivenom efficacy (Antunez et al., 2010; Calvete et al., 2010; Fahmi et al., 2012; Gutierrez et al., 2008, 2010; Huang et al., 2015; Jorge et al., 2015; Makran et al., 2012; Pla et al., 2014; Saviola et al., 2015; Villalta et al., 2012) and for the improvement of antivenom potency and paraspecificity (Fahmi et al., 2012; Goncalves-Machado et al., 2015; Gutierrez et al., 2009, 2013; Makran et al., 2012; Petras et al., 2011; Pla et al., 2014). In addition, the antivenomics approach can also be used to investigate immunological profiles of venoms and the responses to antivenom therapy as a result of interspecies variation (Gutierrez et al., 2013; Makran et al., 2012; Pla et al., 2012), geographic distribution (Fahmi et al., 2012; Goncalves-Machado et al., 2015), age-related venom diversity (Saviola et al., 2015) and finally, to investigate the variable immune response in horses (Villalta et al., 2012).

Antivenomics can be studied primarily by two methods. The first method, so called 'first generation' antivenomics, involves the incubation of venom and antiserum/antivenom in solution. This is followed by addition of either a secondary antibody to completely precipitate the antigen-antibody complexes (Boldrini-Franca et al., 2010; Calvete et al., 2009, 2010, 2011; Fernandez et al., 2011; Gutierrez et al., 2008, 2010; Lomonte et al., 2008; Nunez et al., 2009) or IgG-binding Protein A/G coupled to Sepharose beads to remove antibody complexes (Antunez et al., 2010; Calvete et al., 2012; Fernandez et al., 2011; Petras et al., 2011). The precipitate contains venom proteins bound to the antivenom antibodies while

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the supernatant contains venom proteins that failed to bind to any antibody. The second method, termed 'second generation' antivenomics (Fahmi et al., 2012; Goncalves-Machado et al., 2015; Gutierrez et al., 2013; Huang et al., 2015; Jorge et al., 2015; Makran et al., 2012; Pla et al., 2012, 2014; Saviola et al., 2015; Villalta et al., 2012), uses an antibody affinity column to capture only those venom proteins that bind specifically to the immobilized antibody. In both cases, both the proteins that specifically bind as well as those that fail to bind to the antiserum/antivenom antibodies can be fractionated, usually by RP-HPLC and SDS-PAGE, and identified by proteomics methods (Fahmi et al., 2012; Goncalves-Machado et al., 2015; Gutierrez et al., 2013; Huang et al., 2015; Jorge et al., 2015; Makran et al., 2012; Pla et al., 2012, 2014; Saviola et al., 2015; Villalta et al., 2012).

The advantages of 'second generation'antivenomics using affinity chromatography described by Calvete and his group include the ability to quantitate the extent of antibody binding to each venom protein (Gutierrez et al., 2013; Pla et al., 2012, 2014; Villalta et al., 2012). The venom proteins can then be grouped as either completely bound, unbound or partially bound to the antibody column. Conclusions concerning the immunogenicity (for a homologous venom) or extent of cross-reactivity (for a heterologous venom) can then be drawn. The method can be effectively used with either IgG or $F(ab')_2$ antibodies, and the immunoaffinity column can be reused repeatedly for many cycles (Pla et al., 2012).

However, there are various parameters of immunoaffinity chromatography, e.g. ligand density, the binding and elution conditions, the amount of venom loaded to the column and the relative abundance of some venom proteins, that can affect the binding of venom proteins to an antibody affinity column. These parameters may lead to different chromatographic results and ultimately to differing conclusions as to immunogenicity and/or cross-reactivity of venom proteins. In this work, we report the effect that changes in several parameters of immunoaffinity chromatography have on the binding and dissociation of venom proteins to an antibody affinity column.

2. Materials and methods

2.1. Chemicals and biochemicals

Chemicals were reagent grade and were obtained from Sigma Chemical Co, St. Louis, MO, USA. *N*-hydroxy succinimide (NHS)-activated Sepharose 4 Fast Flow was from GE Healthcare, NJ, USA. Thai cobra (*Naja kaouthia*, NK) venom, milked from dozens of adult snakes and in lyophilized form, and mono-specific equine antiserum (AS) against NK were purchased from Queen Saovabha Memorial Institute (QSMI), Bangkok. Equine polyspecific antiserum against 6 elapid species of Asia (NK, *Naja sputatrix, Naja philippinensis, Naja atra, Bungarus candidus* and *Bungarus multicinctus*) were produced in our laboratory (Ratanabanangkoon et al., 2016). All antisera were aliquoted and stored at −20 °C until used.

2.2. Fractionation of IgG from horse plasma

Fractionation of IgG from each of the antisera by caprylic acid (CA) precipitation was carried out as previously described (Eursakun et al., 2012). Briefly, the normal horse plasma and the plasma from venom immunized horses were 2-fold diluted with distilled water and adjusted to pH 5.5 with 0.875 M acetic acid. CA solution (100%) was slowly added to the diluted plasma to a final CA concentration of 3% with vigorous stirring. Stirring was continued for 1 h at room temperature. The particulate was removed by filtration through 0.45 μm filter and the CA was removed by dialysis against distilled water. The IgG purity and concentration were determined by non-reducing SDS-PAGE and protein assay (Lowry et al., 1951) respectively.

2.3. Affinity chromatography of NK venom on IgG-Sepharose columns

2.3.1. Preparation of horse IgG-Sepharose

Preparation of immunochromatographic matrix has been previously described (Pla et al., 2012). Briefly, NHS-activated Sepharose 4 Fast Flow (3 ml) was washed twice with 15 matrix volumes of ice cold 1 mM HCl prior to washing twice with 2 matrix volumes of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Thereafter, the matrix was incubated at 4 °C overnight with either purified IgG of horse pre-immunized normal serum (nIgG) or horse antiserum IgG (asIgG) which was in 1 matrix volume of the coupling buffer. After rinsing off the uncoupled IgG with 2 matrix volumes of the coupling buffer, the non-reacting NHS groups were blocked by incubation with 2 matrix volumes of blocking buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) at 4 °C, overnight. The affinity columns were washed alternatively at high and low pHs using 3 matrix volumes of 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.5 and 3 matrix volumes of 0.1 M Tris–HCl buffer, pH 8.5. This washing step was repeated 6 times. Then the columns were equilibrated with 3 matrix volumes of the binding buffer termed PBS^+ (PBS containing 0.5 M NaCl and 0.05% NaN_3, pH 7.0) for further experimentation, or washed with 3 matrix volumes of 20% ethanol in PBS for column storage at 4 °C. The amounts of pre-coupled and uncoupled IgG were measured by Lowry's protein assay. The immobilized IgG on the Sepharose bead and ligand density (IgG mg/ml bead) were calculated. The volume of the bead was measured in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3).

2.3.2. Application of snake venom proteins and elution of bound proteins from the IgG Sepharose column

After the columns were equilibrated with PBS^+, crude NK venom (20–160 μg) in 1 matrix volume of PBS^+ was incubated with the IgG coupled Sepharose matrix (nIgG or asIgG column) at 4 °C overnight. The unbound proteins were washed off with 5–6 matrix volumes of PBS^+ and the unbound fractions (1 ml each) were collected. The bound protein was eluted with 5–6 matrix volumes of acid buffer (0.1 M glycine-HCl, pH 2.5) or chaotropic salt (ice-cold 3 M NaSCN). In case of elution with acid, each of the eluted fractions (1 ml) was immediately neutralized with 1 M Tris–HCl, pH 9.0, while the eluent of 3 M NaSCN was diluted with distilled water. The column was immediately washed twice with 5 column volumes of gradient pH of PBS (pH 4, 5, 6, 7) and stored at 4 °C in PBS containing 20% ethanol. The eluted venom proteins were detected spectrophotometrically at 280 nm, the protein fractions were then pooled and assayed for protein by methods described by Lowry et al. (1951).

2.4. Protein separation by reverse phase-HPLC coupled with 1D SDS-PAGE

Both the bound and unbound protein fractions were separately dried in Speed Vacuum, re-dissolved and subjected to C_{18} (0.4 cm × 25 cm, 5 mm particle size, 300 Å pore size) RP-HPLC column (Waters-Micromass., MA, USA) and a High Pressure Gradient System coupled with photodiode array detector and micro-autosampler. The flow-rate was set to 1 ml/min and the column was developed with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B), isocratically (5% B) for 10 min, followed by 5–25% B for 20 min, 25–30% B for 35 min, 30–40% for 65 min, and 40–70% for 30 min. Protein detection was