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RESEARCH PAPER

Identification of proteins interacting with ammodytoxins in *Vipera ammodytes ammodytes* venom by immuno-affinity chromatography

Marija Brgles · Tihana Kurtović · Lidija Kovačič · Igor Križaj · Miloš Barut · Maja Lang Balija · Günter Allmaier · Martina Marchetti-Deschmann · Beata Halassy

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Abstract In order to perform their function, proteins frequently interact with other proteins. Various methods are used to reveal protein interacting partners, and affinity chromatography is one of them. Snake venom is composed mostly of proteins, and various protein complexes in the venom have been found to exhibit higher toxicity levels than respective components separately. Complexes can modulate envenomation activity of a venom and/or potentiate its effect. Our previous data indicate that the most toxic components of the *Vipera ammodytes ammodytes* (*Vaa*) venom isolated so far—ammodytoxins (Atxs)—are contributing to the venom's toxicity only moderately; therefore, we aimed to explore whether they have some interacting partner(s) potentiating toxicity. For screening of possible interactions, immuno-affinity chromatography combined with identification by mass spectrometry was used. Various chemistries (epoxy, carbonyldiimidazole, ethylenediamine) as well as protein G functionality were used to immobilize antibodies on monolith support, a Convective Interaction Media disk. Monoliths have been demonstrated to better suit the separation of large biomolecules. Using such approach, several proteins were indicated as potential Atx-binding proteins. Among these, the interaction of Atxs with a Kunitz-type inhibitor was confirmed by far-Western dot-blot and surface plasmon resonance measurement. It can be concluded that affinity chromatography on monolithic columns combined with mass spectrometry identification is a successful approach for screening of protein interactions and it resulted with detection of the interaction of Atx with Kunitz-type inhibitor in *Vaa* venom for the first time.

Keywords Affinity chromatography · Protein–protein interactions · Ammodytoxins · Monoliths · Mass spectrometry

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M. Brgles (✉) · T. Kurtović · B. Halassy
Centre for Research and Knowledge Transfer in Biotechnology, University of Zagreb, Rockefellerova 10, 10000 Zagreb, Croatia
e-mail: mbrgles@gmail.com

L. Kovačič · I. Križaj
Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

I. Križaj
Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva 5, 1000 Ljubljana, Slovenia

I. Križaj
Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins, Jamova 39, 1000 Ljubljana, Slovenia

M. Barut
BIA Separations, Mirce 21, 5270 Ajdovščina, Slovenia

M. Lang Balija
Institute of Immunology, Rockefellerova 2, 10000 Zagreb, Croatia

G. Allmaier · M. Marchetti-Deschmann
Institute of Chemical Technologies and Analytics, Vienna University of Technology, 1060 Vienna, Austria

Introduction

Protein–protein interactions (PPIs) are important for the majority of biological functions and mapping of all protein

interactions of a living system (interactome) makes one of the exciting "omics" fields—the so-called interactomics [1]. One of the most widely and successfully used methods for assessment of interaction networks is the yeast two-hybrid system which has been used in the automated mode for comprehensive studies of interactomes of various model organisms [2, 3]. Another powerful method for identification of PPIs is tandem affinity purification (TAP) method combined with mass spectrometric identification. Various TAP tags have been developed since the method has originally been introduced that can be used for isolation of highly pure proteins expressed at low natural levels and also their interacting partners [4–8]. Conventional affinity chromatography without the TAP tag, using immobilized proteins, e.g., antibodies against a target protein, is a very useful tool for a simple and rapid complex isolation [9, 10]. In addition, affinity chromatography is also used in other aspects of proteomics such as reduction of sample complexity prior to 2D gel electrophoresis or mass spectrometric experiments [11, 12].

Vipera ammodytes ammodytes (*Vaa*) snake venom proteome comprises more than 100 different proteins that can be grouped into nine protein families [13]. Among the most toxic components of the *Vaa* venom isolated so far are ammodytoxins (Atxs) which are presynaptically neurotoxic secretory phospholipases A_2 ($sPLA_2$). There are three known isoforms of Atx—A (the most toxic), B, and C—all consisting of 122 amino acids and exhibiting all structural elements characteristic for the group IIA $sPLA_2$s such as seven disulfide bridges, a Ca^{2+} binding loop, a His/Asp catalytic dyad, and the C-terminal extension [14]. Atxs have been shown to interact and form homodimers [15] and also most probably heterodimers [16]. In addition to Atxs in the *Vaa* venom, there are also non-toxic $sPLA_2$s—ammodytins I_1 and I_2 ($AtnI_1/I_2$)—with 68 % sequence identity to Atxs and a myotoxic $sPLA_2$ homologue ammodytin L (AtnL) with 74 % sequence identity to Atxs. Due to the high sequence identity (>96 %), Atxs A, B, and C are mutually immunologically cross-reactive, but the anti-Atx antibodies do not recognize $AtnI_1/I_2$ as shown previously [17].

After snake bite, proteins from snake venom interact with various proteins in the victim thereby disrupting their function and inducing disturbance of homeostasis by affecting blood coagulation, blood pressure regulation, transmission of the nervous or muscular impulse, and other. Atxs act as presynaptic neurotoxins, blocking neuromuscular transmission in vertebrate nerve terminals of peripheral motoneurons. In this process, the interaction with neuronal proteins, e.g., R25 and R180, may be involved [18]. High affinity interaction with soluble protein from nerve tissue and calmodulin has also been confirmed [19] as well as interaction with 14-3-3 γ and ε protein isoforms [20]. In addition, proteins in the snake venom have been found to interact also with each other thereby enhancing lethal potency of the venom. Complexes encompassing $sPLA_2$s are very versatile, formed through covalent and mostly non-covalent interactions, resulting in homodimers, heterodimers, and heterooligomers [21]. Example of a covalent heterodimer is β-bungarotoxin formed by $sPLA_2$ and a Kunitz-type serine protease inhibitor (linked by a disulfide bridge) from the venom of *Bungarus multicinctus* [22]. Crotoxin from the venom of *Crotalus durissus terrificus* is a non-covalent heterodimer formed by a $sPLA_2$ and a $sPLA_2$-derived protein [23]. Another example of two diverse $sPLA_2$ molecules non-covalently linked into a complex (vipoxin) is found in *Vipera ammodytes meridionalis* venom [24]. Complexes formed by non-covalent interaction of three and five different $sPLA_2$ molecules are reported for venoms of *Oxyuranus s. scutellatus* and *Pseudonaja textilis* venom, respectively [25, 26]. There is also one trimeric complex named reprotoxin from the venom of *Daboia russelii* described in the literature, encompassing a $sPLA_2$, a serine protease, and a trypsin inhibitor [27]. Snake venom is a rich source of serine proteinase inhibitors and trypsin inhibitor and chymotrypsin inhibitor both belong to non-neurotoxic Kunitz/BPTI (bovine pancreatic trypsin inhibitor) group [28]. Recently, a complex between Kunitz-type and a $sPLA_2$-like protein from the venom of *Micrurus tener tener* has been described [29, 30]. All these examples demonstrate interesting interacting tendencies of snake venom proteins, especially $sPLA_2$ molecules, resulting in regulation of their function and toxic potential.

Atxs have been the most toxic components identified so far in the *Vaa* venom. However, recently we have shown that they contribute to the whole venom toxicity tested in mice only moderately [31]. We hypothesized that the explanation might lie in the possibility that they exert much stronger toxic effects in the whole venom due to complex formation with other, yet undiscovered protein partners. Therefore, the aim of our work was to identify interacting partners of Atxs in the venom of *Vaa*. We used immuno-affinity chromatography based on anti-Atx antibodies coupled to monolithic support for isolation of Atxs and their interacting partners from the venom. Monolithic columns were chosen due to their well-known benefits for isolation of large biomolecules (here complexes) over other conventional chromatographic matrices [32]. Since there are no data on the use of monoliths in PPIs identification, we tested three activated chromatographic materials for coupling of antibodies and compared them with corresponding antibody-free monolithic columns and a monolithic column carrying a completely unrelated antibody. Isolated proteins were analyzed by sodium

dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and identified by means of matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).

Materials and methods

Materials

Iodoacetamide (IAA), 1,4-dithio-DL-threitol (DTT), α-cyano-4-hydroxycinnamic acid (CHCA), Coomassie Brilliant Blue R250, and all peptide standards were from Sigma (St. Louis, MO, USA). Trypsin (proteomics grade) was obtained from Roche Diagnostics (Mannheim, Germany). All organic solvents were of analytical or LiChrosolv grade from Merck (Darmstadt, Germany) and ultrapure water was obtained by Simplicity purification system (Millipore, Billerica, MA, USA). All other chemicals were from Sigma or Kemika (Zagreb, Croatia). *Vaa* venom and rabbit polyclonal antibodies against Atxs and Chi were produced at the Institute of Immunology (Zagreb, Croatia) as described previously [17].

Affinity chromatography

For affinity chromatography, Convective Interaction Media (CIM) disks (BIA Separations, Ajdovščina, Slovenia) were used for ligand coupling: carbonyldiimidazole (CDI), ethylenediamine (EDA), and epoxy-activated, protein G-derivatized CIM disk. Monolithic columns with diameter of 12 mm, length of 3 mm (V=0.34 mL), pore size of 1.5 μm in diameter, and approximately 62 % porosity were used. Coupling of anti-Atx antibodies was performed according to the manufacturer's protocols. When antibody was bound to protein G disk, cross-linking was performed to prevent dissociation of the antibody. Column materials that served as blank were hydroxylated and blocked epoxy-activated column material as well as venom-non-related antibody coupled to epoxy-activated column material. Venom was dissolved in the binding buffer (50 mM phosphate buffer, pH 7.0 with or without 0.5 M NaCl as given in the text) in a concentration of 1 mg/mL and applied to the column (3 mg/run) using a peristaltic pump P 50 (GE Healthcare, Uppsala, Sweden) at 1 mL/min. Column was washed with the binding buffer and then eluted first with 1 M NaCl in binding buffer (elution fraction 1, EF1) and then with 0.1 M citric acid (elution fraction 2, EF2). After several runs, fractions were pooled, concentrated, and transferred to binding buffer (without NaCl) using centrifugal filter devices with a nominal molecular weight limit of 3 kDa (Sarstedt, Nümbrecht, Germany). Affinity columns were found to be functional for more than 3 years (stored at 4 °C in binding buffer with sodium azide). Determination of protein concentrations was done spectrophotometrically [33].

Electrophoresis and detection

Electrophoresis under denaturating conditions was performed using 4–12 % Bis-Tris precast gels, using MES running buffer, on an XCell Sure Lock system from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions. Detection of protein bands was performed using acidic Coomassie Brilliant Blue R250 solution or by Western blotting (see below).

Western blotting

After electrophoresis proteins were electro-blotted onto a PVDF membrane in an XCell II Blot Module according to the manufacturer's procedure (Invitrogen), time of transfer was 1 h. The blocking was performed with 5 % (w/v) non-fat milk in PBS/T buffer for 2 h. After blocking, the membrane was first incubated with anti-Atx serum (25,000-fold diluted) and then with HRP-anti-rabbit IgG (10,000-fold diluted) at 37 °C for 1 h. ECL plus Western Blotting Detection System was used for detection, according to the manufacturer's instructions (GE Healthcare).

For far-Western dot-blot, the protein of interest was blotted onto the membrane and then the membrane was blocked with non-fat milk, probed with the bait protein (5–25 μg/mL), and incubation with antibodies and detection was performed the same as for direct Western blotting.

Sample preparation for MALDI-MS

In-gel digestion was performed by cutting out protein gel bands from the polyacrylamide gel. Bands were washed twice with water followed by water/acetonitrile (ACN) 1:1 (v/v), 15 min each, and 100 μL each wash. After adding ACN, the gel pieces have shrunk, ACN was removed, and gels were rehydrated in 0.1 M NH_4HCO_3 (50 μL) for 5 min. Then an equal volume of ACN was added and incubated for 15 min. After that, gel pieces were dried and rehydrated in 50 μL of 10 mM DTT solution (in 0.1 M NH_4HCO_3) for 45 min at 50 °C. After reduction, samples were alkylated using 50 μL of 54 mM IAA (in 0.1 M NH_4HCO_3) for 30 min in the dark. After that, the alkylation solution was removed and gel pieces were washed with 100 μL of 0.1 M NH_4HCO_3 for 5 min. This was followed by addition of an equal amount of ACN, 15 min incubation, and drying. Gel pieces were rehydrated in 40 μL of trypsin solution (protein amount was visually estimated and 10 ng of trypsin/1 μg of protein was added) and digested

overnight at 37 °C. Alternatively, after 1 h digestion at 37 °C, samples were further digested for 10 min in a domestic microwave oven at 750 W. After digestion, 50 mM NH_4HCO_3 (50 μL) was added and incubated for 15 min followed by addition of the same amount of ACN and 15 min incubation. This solution was recovered. Extraction was repeated two times with 50 μL of 1 % HCOOH and ACN (1:1, v/v). All extracts were pooled, dried, and purified using ZipTips C_{18} (Millipore, Billerica, MA, USA). Purified peptide solutions were dried and stored at 4 °C until analysis. Prior to analysis, peptides were dissolved in 5 μL of CHCA solution [3 mg/mL, in 0.1 % TFA/ACN (50:50, v/v)] and two spots, each 2 μL, were spotted onto the MALDI-MS target followed by drying at room temperature.

MALDI-TOF MS analysis

Measurements were performed on an AXIMA TOF^2 and AXIMA CFR^+ instrument (Shimadzu–Kratos Analytical, Manchester, UK). The instruments are equipped with 20 Hz nitrogen lasers (337 nm) and were operated in the positive ion mode applying an accelerating voltage of 20 keV. Delayed extraction was used for all experiments to optimize resolution (optimal delay time was set according to the m/z range of interest). Typically, peptide mass fingerprint (PMF) mass spectra were acquired by averaging approximately 300 unselected single laser shots. For post-source decay (PSD) experiments, the laser fluent was slightly increased above the threshold to promote fragmentation and 3,000–5,000 unselected laser shots were accumulated.

Raw data generated on AXIMA instruments were converted into mzXML files, processed using mMass [34], and searched against SwissProt database and NCBInr. Searches were performed against Chordata, with the following fixed parameters: precursor ion mass tolerance±0.6 Da, product ion mass tolerance of±1.2 Da, two missed trypsin cleavages, carbamidomethylation of Cys, and with variable modification: ammonia loss from N-terminal Cys. Some deviations from expected peptide masses such as Asn deamidation and ammonia loss from N-terminal carbamidomethylated Cys were confirmed by fragment ion analysis.

Surface plasmon resonance assay

Surface plasmon resonance (SPR) experiments were performed at 25 °C similarly as described [20]. All solutions were passed through a 0.22-μm filter and degassed before each experiment. Recombinant AtxA was immobilized on flow cell 2 of a CM5 sensor chip (GE Healthcare) while the flow cell 1 was mock-immobilized with ethanolamine and used as a reference surface. SPR experiments were performed in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM $CaCl_2$ 0.005 % (w/v) Tween 20 (buffer A) at 30 μL/min using a Biacore X system (GE Healthcare). The interaction between Chi and the chip-immobilized AtxA was studied by injecting 20-μL solutions of Chi (concentrations from 154 μM to 70 nM by dilution factor 2) in buffer A over the chip and following the dissociation for 60 s. Between consecutive injections, the chip was regenerated three times with 5 μL of 10 mM NaOH. The kinetic constants, K_s, K_d, and K_D for the interaction of Chi with immobilized AtxA were determined by using the Biaevaluation 3.2 software and GraphPad Prism. The experimental curve was fitted according to a simple one-to-one model of interaction.

Assay of lethal toxicity

All animal experiments were done according to Croatian Law on Animal Welfare (2013) which strictly complies with EU Directive 2010/63/EU. The lethal toxicity of fractions EF1 and EF2, as well as of pure AtxA, pure Chi, and their combinations, was determined according to the method of Theakston and Reid with experimental details described previously [35, 36]. The toxicity was accordingly expressed as median lethal dose (LD_{50}), i.e., the amount (in micrograms per kilogram) of tested material causing the death in half of the mice population used (four mice per group).

Preparation of venom component-specific antisera

Anti-EF1 and anti-EF2 rabbit sera were prepared by immunizing rabbits according to procedure described by Kurtović et al. [31]; only antigens for immunization were EF1 or EF2 (100 μg/rabbit/immunization), respectively.

Lethal toxicity neutralization assay

The potential of rabbit sera to neutralize the lethal toxicity of venom (protective efficacy) was determined in mice according to Ph. Eur. 01/2008:0145, with minimal modifications as described [17]. The toxicity neutralization potency (R) of each serum was expressed as the number of LD_{50} venom doses that can be neutralized with 1 mL of undiluted serum and was calculated from the following equation: $R = (Tv - 1)/ED_{50}$, where Tv represents the number of LD_{50} inoculated per mouse and ED_{50} the quantity of undiluted serum (in microliters) that neutralizes the lethal toxicity of the applied venom dose in 50 % of mice (the result of the assay).

Quantification of anti-Atx and anti-H IgGs in rabbit sera

The quantity of anti-AtxA immunoglobulin G (IgG) in rabbit sera was determined by ELISA as described [31], and the results are given in arbitrary unit per milliliter (AU mL^{-1}).

Results

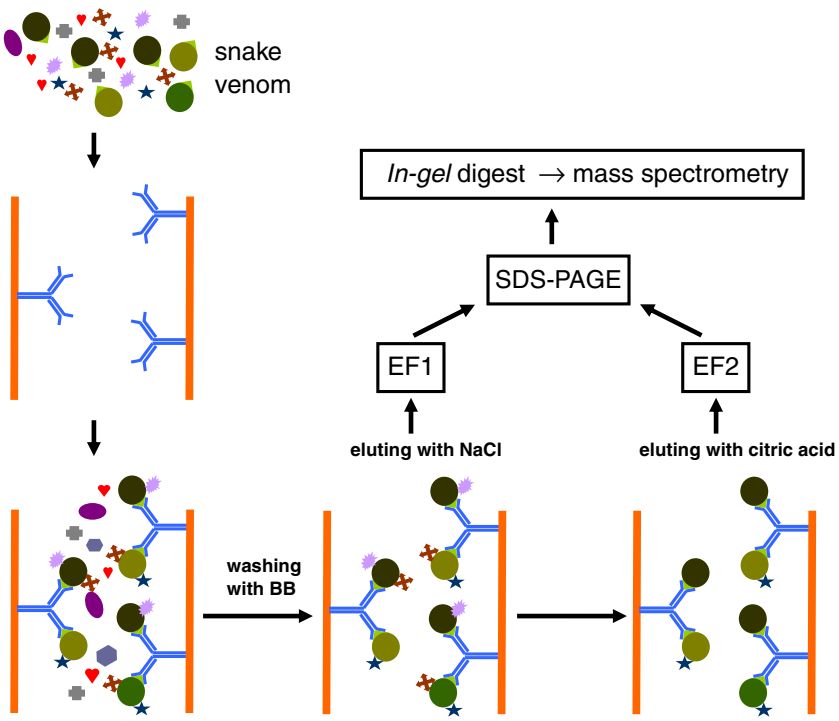
Identification of Atx-complexing protein candidates in *Vaa* venom by affinity chromatography

Anti-Atx antibodies were successfully immobilized on epoxy and protein G CIM disks with around 1 mg of antibodies per disk. Affinity chromatography of *Vaa* venom yielded two protein fractions, EF1 (proteins eluted with 1 M NaCl) and EF2 (proteins eluted with 0.1 M citric acid pH 2) (Scheme 1). According to SDS–PAGE analysis, proteins eluted from anti-Atx-epoxy and anti-Atx-protein G columns were the same (data not shown) so these disks were used stacked together in one column (to increase capacity per run). Protein fractions EF1 and EF2 were analyzed by SDS–PAGE and protein bands were cut from the gel, enzymatically cleaved with trypsin, and analyzed by PMF MS and PSD MS (shown in Fig. 1a). Some *Vaa* protein sequences are deposited in the databases and several proteins (Chi, Atxs, AtnIs, and AtnL) were identified using these sequences, while the rest of them [C-type lectin, CRISP, SP, metalloproteinase (MP)] were identified via homology with other snake proteins (MS identification data are summarized in Table S1, Electronic Supplementary Material). EF1 from the anti-Atx affinity column contained protein bands corresponding to Atxs, AtnL, $AtnI_1/I_2$, and eight additional bands. EF2 contained mostly Atxs, AtnL, $AtnI_1/I_2$, and in addition two faint protein bands that were identified as Chi and MP. Metalloproteinase was confirmed in EF1 and EF2 also by Western blot (Fig. S1, Electronic Supplementary Material).

We searched for Atx-complexing partner within EF2 proteins, namely Chi, AtnIs, AtnL, and MP, since it is to be expected that these are retained by stronger interactions in comparison to EF1 proteins. The existence of toxic molecules or possibly complexes in the EF2 was additionally supported by results of the toxicity neutralization studies. Collected EF1 and EF2 venom fractions were used to immunize rabbits and produce anti-EF1 and anti-EF2 sera. The ability of these sera to neutralize the whole venom toxicity was tested in comparison to the neutralization power of the anti-venom and anti-Atx sera, which were produced by immunization of rabbits with the whole venom or with the pure ammodytoxins, respectively, and characterized as described [31]. Both anti-EF1 and anti-EF2 sera had similar and small quantities of anti-Atx antibodies determined by ELISA; however, they differed significantly in their ability to neutralize the whole venom toxicity (Table 1). Anti-EF2 serum was minimum twice as more protective than anti-EF1 serum. Of importance is that it was even more protective than anti-Atx serum, containing the highest quantity of anti-Atx IgGs.

To investigate whether some of the proteins in the EF2 were retained to the affinity column due to non-specific interactions, we performed the negative control experiments. The same chromatographic procedure was performed, only using, as a stationary phase, either the epoxy-activated disk coupled with an unrelated antibody that was raised against virus antigen (Fig. 1b) and was proved not to recognize venom proteins by Western blot (data not shown) or plain column without an antibody (Fig. 1c). Elution pattern of proteins in EF1 (Fig. 1b) from Atx-non-related affinity column was similar to the pattern from an anti-Atx column (Fig. 1a), but MS analysis revealed that the major protein bands were AtnL and $AtnI_1/I_2$ (AtnL, $AtnI_1/I_2$, and Atxs share many common peptides due to high sequence homology, but those unique for Atxs were not detected). Other proteins although similar in molecular masses to those from EF1 from anti-Atx column (Fig. 1a) could not be identified except for Chi. Of importance, in fraction EF2 only AtnL was identified unambiguously, indicating specifically strong non-specific interaction between this protein and column, that resisted elution with 1 M NaCl used for EF1. In short, among proteins isolated from the venom on this column are AtnL, $AtnI_1/I_2$, and Chi (only AtnL in the EF2) indicating that at least in part these proteins might be retained non-specifically by the column matrix. Chromatography of venom using this control column revealed that strong non-specific interactions are occurring between the AtnL and the matrix since it was detected as the only strong protein band in EF2. A similar pattern was observed with the blocked epoxy column without any antibody (Fig. 1c) with the only difference that Atxs were confirmed in EF1 and EF2 also. In short, among EF2 proteins from affinity column, AtnL is definitely prone to make strong non-specific interactions with the column matrix.

To understand whether some of the EF2 proteins were strongly retained on the column due to cross-reactivity to ammodytoxins, i.e., due to the specific binding to anti-Atx antibody, and not by being in complex with Atx, the specificity of anti-Atx antibodies was investigated in detail. Western blot of the whole venom under non-reducing conditions [venom was not reduced to potentiate possible interactions since almost no recognition of Atxs was found when reduced venom proteins were transferred to membrane (Fig. S1, Electronic Supplementary Material)] with anti-Atx antibodies (Fig. 2a) showed their specific recognition of Atxs (14 kDa), proteins of apparent mass of 28 kDa (possible Atx dimers and/or CRISP), and very weakly 35-kDa proteins (according to mass possibly serine protease). They also weakly recognized proteins at 60 and 110 kDa (which can presumably correspond to metalloproteinases since they are the only protein family with such mass in the venom). The same experiment showed that anti-Atx antibodies do not recognize anything below 14 kDa, indicating lack of cross-reactivity between Atx and Chi (7 kDa). Western dot-blot using pure available venom proteins—isoforms of $sPLA_2$s—showed that anti-Atx antibodies recognized Atxs, namely A, B, and C, very faintly AtnL, but do not recognize $AtnI_2$



Scheme 1 Analytical procedure for screening of Atxs' interacting partners

(Fig. 2b). ELISA results (Fig. 2c) additionally confirmed lack of cross-reactivity between Atxs and AtnIs and between Atxs and Chi, and demonstrated that AtnL and Atxs are partially cross-reactive. All presented data indicated that among EF2 proteins, AtnL shares cross-reactivity with Atxs, very weakly MP also, while AtnIs and Chi were not cross-reactive.

Taken altogether, it can be concluded that within EF2 proteins the most probable candidates as Atx interacting partners would be Chi or AtnIs because both were shown to

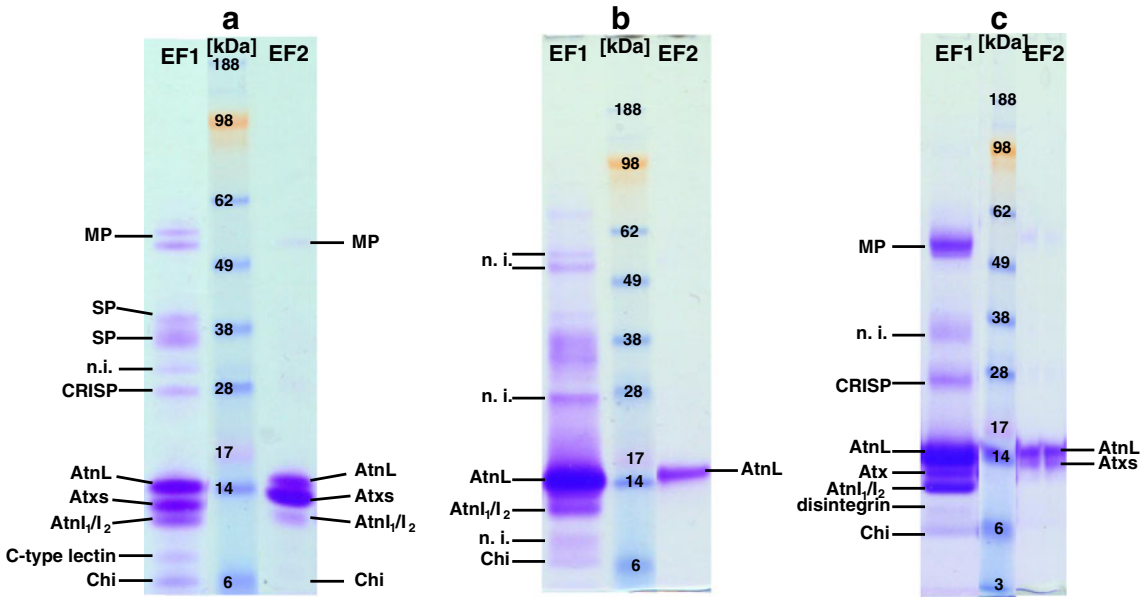


Fig. 1 Chromatographic fractions of snake venom from Atx-specific and control columns. Proteins isolated from snake venom by chromatography using anti-AtxA affinity column (**a**), Atx-non-related antibody affinity column (**b**), and control column (blocked epoxy column without any antibody bound) (**c**). Retained proteins were eluted from the columns by 1 M NaCl (EF1) and citric acid (EF2). SDS–PAGE of protein fractions was performed under reducing conditions, protein bands cut out, and used for MS identification. *n.i.* not identified, *MP* metalloproteinase, *CRISP* cysteine-rich secretory protein, *SP* serine protease, *AtnL* ammodytin L, *AtnI* ammodytin I, *Atxs* ammodytoxins, *Chi* chymotrypsin inhibitor

Table 1 Properties of anti-EF1 and anti-EF2 rabbit sera

Serum	Anti-Atx IgG quantity/AU mL^{-1}	Toxicity neutralization power (R)/LD_{50} mL^{-1}
Anti-EF1	9,677±747	7.09±1.08
Anti-EF2	13,015±510	19.22±4.07
Anti-Atx	66,981±1455	5.13±1.99
Anti-venom	29,469±447	53.76±5.25

lack cross-reactivity with Atx and were not retained in the EF2 in control affinity columns.

Atx–Chi complexing studies

Interaction of Atxs with Chi was tested by far-Western dot-blot. The test was carried out in both directions, i.e., in one case Chi was spotted on the membrane, probed with Atx, and then complexes detected with anti-Atx antibodies; alternatively, Atx was spotted on the membrane, probed with Chi, and complexes detected with anti-Chi antibodies. Interaction between Atx and Chi was detected as positive (Fig. 3). In the same experiment, possible interactions of ammodytoxins with AtnL and AtnIs were tested, but these interactions were not detected (Fig. 3).

Specific interaction between Atx and Chi was confirmed also by SPR analysis. The AtxA-immobilized CM5 sensor chip was used. Solutions of Chi were injected over the chip at different concentrations to obtain sensorgrams. Representative sensorgrams of the four experimental repetitions performed at equilibrium conditions are shown in Fig. 4a. Based on a 1:1 binding model, the binding parameters for the interaction between immobilized AtxA and Chi were calculated and resulted in the K_D value of 1.4±0.9 μM. As the association rate (k_s) between Chi and AtxA was very fast at such conditions, the reliability of obtained result is accordingly lower. Therefore, we determined the K_D also at higher concentrations of Chi, at the so-called steady-state conditions (Fig. 4b). In this case, the K_D value was 9.7±0.4 μM. This time the problem was the aggregation of Chi noticed at higher concentrations. From the SPR experiments, we can conclude therefore that Chi and AtxA associate in the low micromolar range.

Physiological relevance of Atx–Chi complex was tested in mice model with pure AtxA and Chi for comparison, and results are given in Table 2. Chi exhibited no toxicity at all. Atx–Chi complexes were prepared at 1:1 and 1:4 stoichiometry (Atx/Chi) and LD_{50} doses were determined, as well as for EF1 and EF2 fraction and the whole venom. Results showed that LD_{50} of EF1 was of the same order of magnitude as LD_{50} of the whole venom. LD_{50} of EF2 fraction was the same as for pure AtxA, the most toxic form of Atxs, and that Atx–Chi combinations have slightly increased the toxicity of the Atx.

Comparison of different immobilization chemistries in affinity chromatography

Affinity chromatography of snake venom described so far revealed that plenty of non-specific interactions are unavoidable when such a complex mixture, containing also highly basic molecules, is a starting material for chromatography. Thus, we found this fact interesting to use in order to gain knowledge on the differences that could be expected if different chemistries for immobilization of

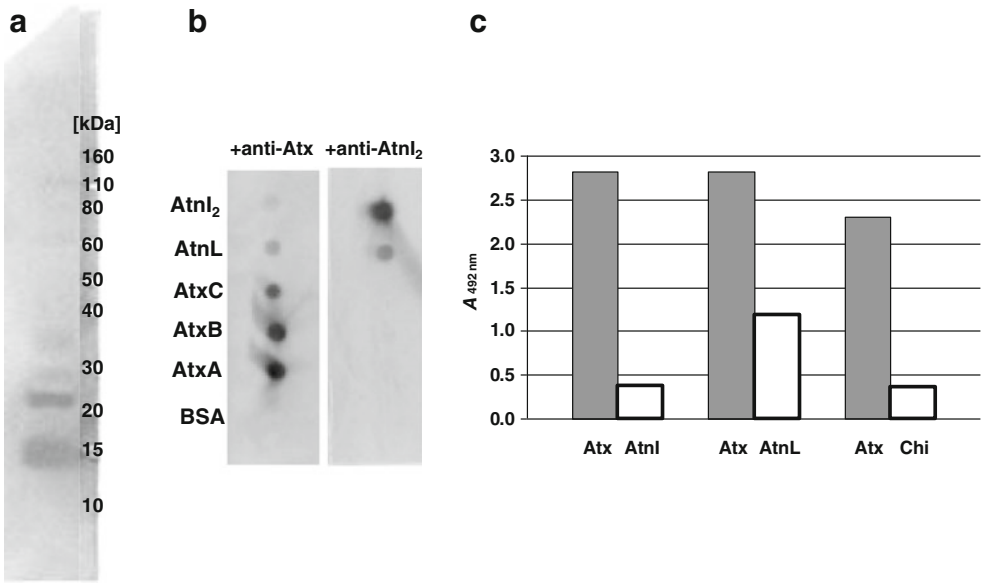


Fig. 2 Specificity of anti-Atx antibodies used for affinity chromatography towards *Vaa* venom and particular venom components. Western blot of proteins in *Vaa* venom detected by anti-Atx antibodies (**a**). Recognition of isolated *Vaa* venom components by anti-Atx and anti-$AtnI_2$ antibodies in Western dot-blot (**b**). Anti-Atx antibody recognition of AtnIs-, AtnL-, or Chi-coated wells in ELISA, in comparison to Atx-coated wells (**c**)

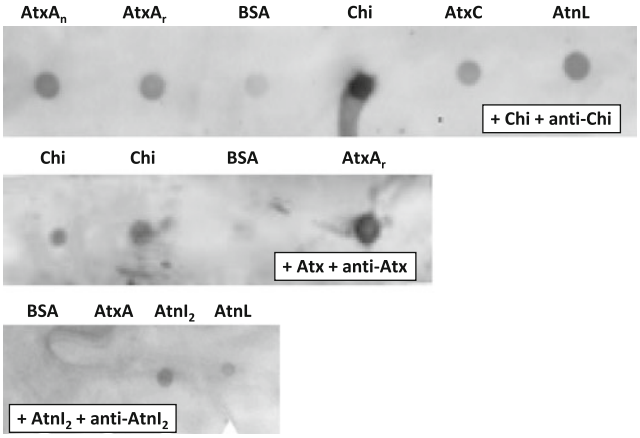


Fig. 3 Screening of possible interactions between Atx and Chi and $AtnI_2$ and venom components by far-Western dot-blot. Proteins spotted on the membrane (denoted above the membrane) are probed with the protein denoted in the *white rectangle* and detected by the corresponding antibody. *Atxn* Atx native, *Atxr* Atx recombinant

antibodies to column were used, and subsequently possible impact on affinity chromatography. Similar to epoxy-activated column described above, anti-Atx antibodies were successfully immobilized on CDI and EDA columns with around 1 mg of antibodies. Blocked epoxy disk (described already in the first section of "Results") and plain hydroxy disk were used as control columns for comparison. Regarding binding capacity of affinity columns, CDI column showed the highest capacity, around 0.5 mg protein/run, and this is around four to five times more than of the other columns used here.

Influence of different ionic strength of binding buffer (0.1 M or 0.5 M NaCl in BB) was also studied. The existence of many protein bands in EF1 fraction of the control columns

Table 2 Toxicity of *Vaa* venom, isolated protein fractions, and proteins in mice

Sample	Toxicity LD_{50}/μg kg^{-1}
Venom	331
EF1	223
EF2	44
AtxA	37
Chi	Not toxic
AtxA + Chi 1:1	33
AtxA + Chi 1:4	33

(Fig. 5a, b) indicated that different molecules from the venom have the ability to non-specifically interact with the column matrix. Among them, particularly strong non-specific interactions with matrix were exhibited by AtnL, followed by Atxs themselves, since these two proteins were the only ones retained to the blank columns even after elution with 1 M NaCl and detected in EF2 in the experiment where 0.1 M NaCl was used in binding buffer. Complete elimination of any non-specific interaction with matrix was achieved when 0.5 M NaCl was used in the binding buffer, as proved by empty EF2 fraction (Fig. 5b). Protein patterns of EF1 fractions eluted from blank columns, when 0.1 M NaCl in binding buffer was used, showed differences (Fig. 5a, lines hydroxy and epoxy). For example, AtnIs and MP were retained more strongly on epoxy column, in contrast to Atx that was in higher quantity retained on hydroxy column. Usage of 0.5 M NaCl in binding buffer resulted in the same EF1 protein patterns from both blank columns (Fig. 5b).

EF1 protein patterns of epoxy and EDA affinity columns were similar, irrespective if 0.1 M NaCl (Fig. 1a compared to

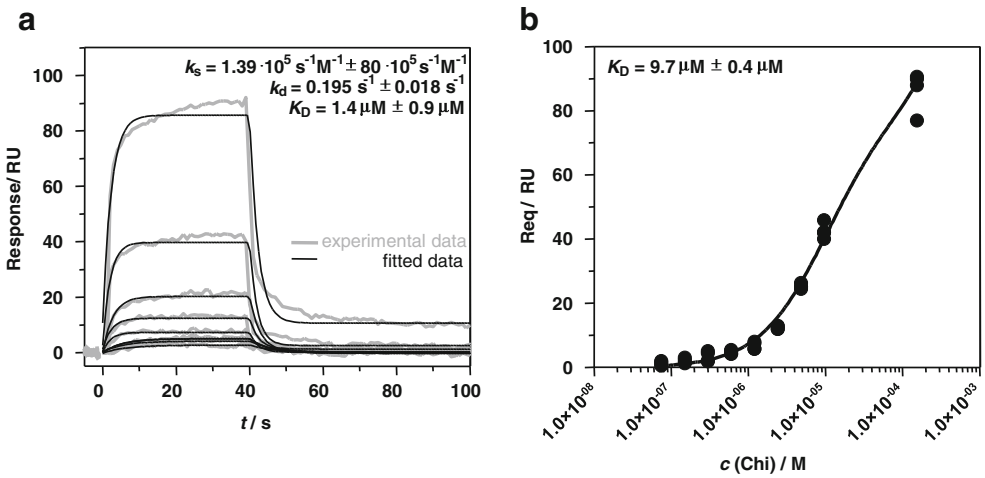


Fig. 4 Chi binds to immobilized AtxA in a low micromolar range. Chi was injected over AtxA immobilized on the CM5 sensor chip in concentrations ranging from 0.07 to 154 μM. The experiment was repeated four times. Representative sensorgrams are shown in (**a**). Experimental data were fitted considering 1:1 binding model. Calculated rate constants, k_s and k_d, gave the K_D value of 1.4±0.9 μM. The rate of association of Chi and AtxA (k_s) was very fast and the reliability of the result was accordingly lower. For this reason, the K_D was also calculated at the steady-state conditions (Req). Assuming 1:1 binding, the K_D obtained in this case was higher: 9.7±0.4 μM (**b**). As the aggregation of Chi was observed at higher concentrations, the true K_D value for the interaction between Chi and AtxA is somewhere between both experimental values

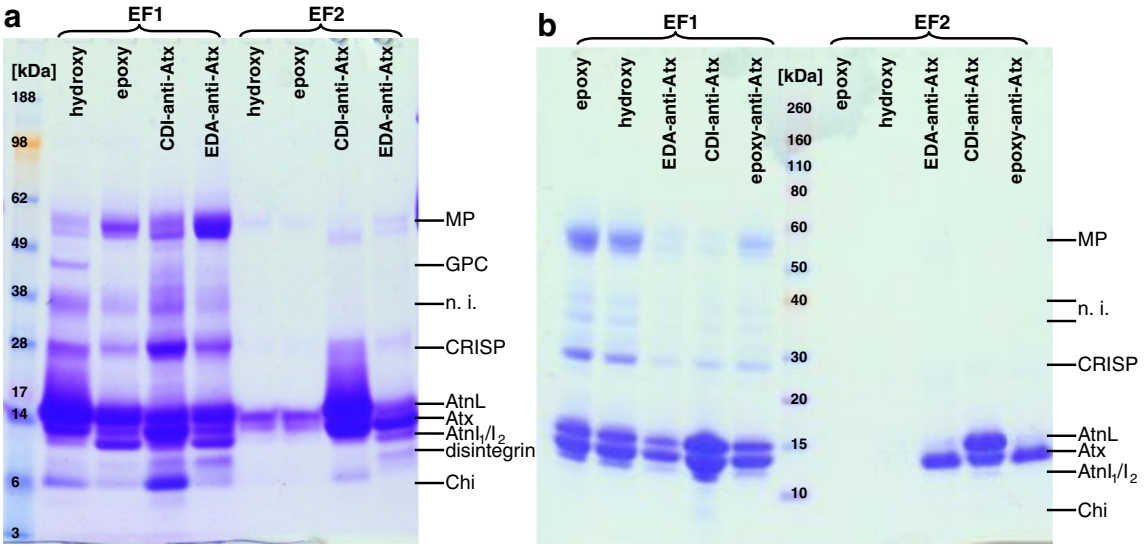


Fig. 5 SDS–PAGE of protein fractions isolated from *Vaa* venom on various specific and non-specific columns (as denoted on each well) eluted with 1 M NaCl (EF1) and citric acid (EF2). Binding buffer was 50 mM phosphate buffer, pH 7.0 (**a**), and 50 mM phosphate buffer, pH 7.0+0.5 M NaCl (**b**). Proteins denoted on the figure were identified by MS. *n.i.* not identified, *GPC* glutaminyl-peptide cyclotransferase, *MP* metalloproteinase, *CRISP* cysteine-rich secretory protein

Fig. 5a) or 0.5 M NaCl (Fig. 5b) was in the BB. CDI affinity column differed from the two by its ability to retain significantly higher quantities of small basic venom components, like AtnL, Atxs, and Chi in EF1 (Fig. 5a, b). Particularly strong interaction was with AtnL, which was eluted in EF2 even in higher quantity that Atx itself, even when 0.5 M NaCl was used in the BB (Fig. 5b).

Discussion

Intermolecular interactions in affinity chromatography and PPIs

The aim of this research was to explore the possibilities of monolithic affinity columns (CIM disks) to identify proteins in the *Vaa* venom that form complexes with Atxs. Affinity chromatography is a powerful technique for isolation of proteins but also for identification of PPIs and other application areas resulting from the specificity of the method [4–12]. The version of the affinity chromatography using TAP tag usually uses two (or more) specific steps resulting in reduced non-specific interaction and elution under native conditions, but influence of the tag on the structure of the bait protein thereby diminishing probability of detection of all existing interactions is unfortunately possible [7]. Conventional affinity chromatography as described and used here is attractive due to its simplicity, but it also has its shortcomings such as non-specific interactions and problems with elution, especially of native proteins. For correct analysis of results, it is very important to consider and keep in mind all interactions involved in PPIs and also in affinity chromatography [37–40]. In antibody-based affinity chromatography, an antibody is covalently linked to the matrix, antigen is bound to the antibody, and proteins interacting with the antigen are bound to antigen and therefore retained on the column. PPIs are controlled by a complex array of intermolecular and intersurface forces, in general consisting of van der Waals interactions, hydrogen bonds, and hydrophobic interactions (i.e., hydrophobic effects) and also contributing induced conformational changes. Since these interactions are very complex, various effects are possible. However, when eluting proteins from the affinity column by increasing ionic strength, it can be expected that van der Waals interactions are disturbed and hydrophobic interactions can possibly be enhanced. Low/high pH, i.e., protonation/deprotonation, breaks hydrogen bonds and disrupts all interactions including groups susceptible to protonation/deprotonation under a given pH. In the case of very strong interactions, denaturation buffers are needed for complete dissociation of antigen from the antibody. An additional point is the possible direct interaction with the column matrix material. The monolith matrix for the affinity columns used in this work is poly(glycidyl methacrylate-co-ethylenedimethacrylate) which has a hydrophobic core and carbonyl groups; both can possibly be engaged in non-specific interactions.

When snake venom is applied to the column, it is expected for the experimental setup used here, with the polyclonal anti-Atx antibodies bound to monolith matrix, that all proteins recognized by covalently linked antibodies (i.e., Atxs and Atx-cross-reactive proteins which are shown in Fig. 2) bind to the column. Also, it is expected that proteins interacting with the bound proteins are retained as well. In addition, venom proteins interacting non-specifically with the column

matrix or any bound proteins are also retained. It is expected that binding forces between proteins recognized by antibodies are strongest of all interactions considered here and require the harshest elution conditions (acidic conditions are used here) whereas breaking bonds between Atxs and interacting partners depends on the strength of the interaction. Accordingly, breaking bonds between Atxs and interacting proteins could occur during elution with increased ionic strength or with acid elution. The problem is that the strength of these interactions of Atxs and their interacting partners could be similar to non-specific interactions of other venom proteins with the matrix or Atxs thereby making their differentiation almost impossible with the given experimental setup. This is a drawback, but nevertheless, the method proved to have applicability despite that.

Since proteins detected in chromatographic fraction eluted with salt (EF1) contained several proteins which were also detected using control columns (Fig. 1), focus was set on proteins eluted with low pH (EF2) which are therefore expected to exhibit stronger interactions. Biological activity of obtained chromatographic fractions was also studied by measuring toxicity neutralizing power of anti-EF1 and anti-EF2 sera and content of anti-Atx antibodies (Table 1). Interestingly, although content of anti-Atx antibodies was only slightly higher in EF2 fraction, the toxicity neutralizing power was more than two times higher in EF2 fraction. This indicates that EF2 contains more toxic molecules in addition to Atxs and/or possibly toxic complexes. In addition to Atxs, EF2 fraction was found to contain Chi, AtnIs, AtnL, and MP. Since all components of EF2 except Atxs are non-toxic, it could be concluded that EF2 contains complexes and that toxicity-neutralizing power comes from the ability of anti-EF2 to neutralize these toxic complexes. MP and AtnL were shown to be at least partially cross-reactive with Atxs and additionally AtnL was found to interact non-specifically with the matrix. Therefore, Chi and AtnIs appeared as likely partners for interaction with Atx. These interactions were tested by far-Western dot-blot (Fig. 3), a method shown as very informative for protein interaction detection [41], and interaction of Atx with Chi was found positive. Atx–Chi interaction was further confirmed by SPR (Fig. 4). Preliminary toxicity experiments in vivo also indicated that Atx–Chi mixture is more toxic than the Atx itself whereas Chi per se was non-toxic to mouse.

Despite the drawbacks of the methodology discussed at the beginning, the affinity chromatography in combination with other immune techniques resulted in fast selection and identification of Chi as the Atx interacting partner, out of the over 100 different proteins being present in the snake venom. A non-covalent complex between a $sPLA_2$ molecule and a Kunitz-type inhibitor has been characterized before in *Micrurus tener* venom [29]. The complex found in the venom of *M. tener* was toxic whereas the components by themselves were not [29]. A similarity was also found here, where the complex Atx–Chi is more toxic than one component (Atx) whereas the other is by itself non-toxic. This complex can be added to a series of various examples of snake venom complexes found to exhibit increased toxicity in comparison to constituting components [21–27]. However, this and possible higher order complexes and their roles remain to be further investigated.

CIM disks in affinity chromatography

Due to their large pores and low backpressures, CIM disks are considered as stationary phases especially suited for separation of large and labile biomolecules [32]. Affinity chromatography of snake venom described so far revealed that plenty of non-specific interactions are unavoidable when such a complex mixture, containing also highly basic molecules, is a starting material for chromatography. Therefore, different CIM disks were used, i.e., epoxy, CDI, and EDA-activated as well as protein G-coupled, to compare their performance. The amount of antibodies bound to all activated CIM disks used was around 1 mg, which is in accordance with the published data [42]. First, we used columns containing epoxy-activated and protein G-coupled CIM disks. The capacity of both types of disks was the same (≈0.1 mg total protein/run) and also the same types of proteins were retained by them, as published before [16]. Anti-Atx-CDI disks exhibited the highest capacity of all disks prepared (≈0.5 mg/run), consistent with the literature [42, 43]. Control disks, i.e., those without any antibody bound, blocked epoxy and hydroxy column material, exhibited capacities below 0.1 mg/run. Comparison of epoxy and hydroxy control columns revealed different non-specific binding of venom proteins (EF1 profiles, Fig. 5a); however, this was diminished by higher ionic strength of the binding buffer (EF1 profiles, Fig. 5b). Also, 0.5 M NaCl in binding buffer completely reduced all non-specific interactions in EF2 (Fig. 5b). This indicated that the observed non-specific interactions are largely the result of electrostatic and not hydrophobic forces. The strongest non-specific binding to the column matrix was showed by AtnL. This may be the consequence of the fact that AtnL is the most basic ($pI = 10.4–10.6$) $sPLA_2$ molecule in the snake venom. Its non-specific binding was additionally increased when using CDI for affinity chromatography (Fig. 5b). Epoxy and EDA affinity columns showed similar properties, while CDI showed higher capacity and also higher non-specific binding. Taken all together, one can conclude that epoxy, EDA, and protein G affinity columns can be used for isolation of Atxs and proteins possibly interacting with them, while CDI showed to be less suitable in this particular case due to increased non-specific affinity for basic proteins.

Increase in the ionic strength of the binding buffer diminishes some interactions as expected, but relevant biological interactions can be of lower strength and therefore the ionic strength of the binding buffer should be optimized so as to diminish non-specific and not to destroy weak specific interactions, and this is possible to determine by trial and error process.

Conclusion

Affinity chromatography based on immobilized antibodies combined with mass spectrometry is a straightforward and powerful method combination for identification of PPIs. Weaknesses of the separation method are possible non-specific interactions of proteins in play and with the chromatographic matrix. Nevertheless, this can be reduced to some extent by careful selection of binding and elution buffers, and possibly by using monoclonal antibodies. Monolithic columns used here—epoxy, CDI, EDA-activated, and protein G-functionalized CIM disks—were found to exhibit similarly good performances (with exception of CDI exhibiting highest capacity and higher affinity for basic proteins) and are therefore columns of choice for affinity chromatography and identification of PPIs since they are designed for large biomolecules. The very basic nature of proteins in focus of this work could be the cause of some background proteins due to possible interaction with carbonyl groups of poly(glycidyl methacrylate-co-ethylenedimethacrylate), but other non-specific intermolecular interactions are also possible. However, this relatively simple separation method revealed that Chi is interacting partner of Atxs, the most toxic components of the *Vaa* venom. This was corroborated by orthogonal methods far-Western dot-blot and SPR analysis. Testing of positive interactions detected by any method is recommended because a false-positive result is always possible. However, this requires pure components and cannot always easily be detected using existing methods especially for weaker and/or transient interactions. Interaction of Atxs with Chi in *Vaa* venom is confirmed for the first time and is in line with recently described similar complex of PLA_2 molecule with Kunitz-type inhibitor found in *M. tener tener* venom and covalent complex composed from the same type of molecules in *Bungarus candidus* venom. Furthermore, preliminary data indicate an increase in toxicity of the complex in comparison to pure components. However, the exact physiological effect of this complex in *Vaa* venom and possible other interactions remain to be determined.

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