

Induction of Neutralizing Antibodies against Mutalysin-II from *Lachesis muta muta* Snake Venom Elicited by a Conformational B-cell Epitope Predicted by Blue Star Sting Data Base

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Abstract

Mutalysin-II, from *Lachesis muta muta* snake venom, is an endopeptidase with hemorrhagic activity. To identify a conformational epitope we used Blue Star Sting, the latest version of the web based Sting Millennium Suite, as alternative computational analysis tool to select and design peptides. Pre-selected cut-off values of the accessibility and hydrophilicity parameters were used to select amino acid residues as potential conformational epitopes. A peptide (P117-Y116-C115-Q194-C195-L197-N198-K199-P200-Y5-L48) was manually drawn on Swiss-PDB-Viewer package and synthesized by Fmoc-synthesis. Immunization of rabbits with this peptide induced antibodies that recognized Mutalysin-II and protected against the hemorrhagic factors present in *Lachesis* venom. The Sting Millennium Suite was able to predict conformational epitopes in this class of proteins. Three amino acids (K199, Y5 and L48) were identified as essential in the interaction between the peptide and the neutralizing antibodies.

Keywords: *Lachesis muta muta* venom; Antivenom; Sting millennium; Synthetic peptides; Epitopes; Conformational epitope prediction

Introduction

Snakebites are considered a neglected tropical condition in the World and an important public health problem in Brazil [1]. The pit viper envenomation is generally characterized by severe hemorrhage, blood incoagulability and renal insufficiency. In *Bothrops* sp. and *Lachesis* sp. Snake Venoms Metalloproteinases (SVMPs) are the most important contributors to local and systemic hemorrhage observed in human snakebite victims [2]. The venom of the pit viper *L. muta muta* contains a proteinase, called mutalysin-II (Mut-II) a P-I snake venom metalloproteinase (P-I SVMP) and belongs to a major class of metalloproteinases known as the metzincins [3]. Mut-II is a 22.5 kDa single chained protein with broad substrate specificity and traces of hemorrhagic effects [3,4]. Like other SVMPs, mutalysins proteolytically degrade fibrinogen and other extracellular matrix components such as laminin, fibronectin, and type IV collagen or gelatin [4].

Due to the medical importance of the envenomation caused by the *L. muta muta* and the role played by P-I SVMPs in these accidents, polyclonal and monoclonal antibodies against these zinc-endopeptidases may be considered as highly useful tools to investigate the structural determinants of toxicity and antigenicity/immunogenicity of these molecules. Furthermore, these antibodies could have great potential for the preparation of more efficacious anti-venoms for passive immunotherapy or even for vaccination. Effectively, the monoclonal antibody LmmAbB2D4 [5] and rabbit polyclonal antibodies [6] against Mut-II efficiently neutralize the hemorrhagic effects of whole *L. muta muta* venom and certain *Bothrops* venoms. Prediction and mapping of neutralizing epitopes on these toxic proteins are vital steps to create epitope-based antivenoms design (i.e. antivenoms produced using synthetic peptides corresponding to identified epitopes as immunogens). We have showed that synthetic peptides corresponding to linear and conformational epitopes previously mapped using laboratory methods, are capable of inducing neutralizing antibodies against *L. muta muta* crude venom [6,7].

Although laboratory techniques, such as Phage display [8] and Spot method [9], are effective in identifying epitopes, they are expensive and time consuming. Therefore, computational analysis can provide a rapid method to identify functional epitopes using data routinely collected from toxins. STING Millennium Suite (SMS) [10] is web-based, publicly available software developed to aid researches in their quest for translating information about the structures of macromolecules into knowledge. Using SMS it is possible to analyze the sequence/structure relationship, quality of the structure, nature and volume of intra and inter chain atomic contact and the relative conservation of amino acids at the specific sequence position based on multiple sequence alignment to mention only a few [11]. In the present investigation, we used STING Millennium Suite (SMS) as an alternative computational analysis tool to design and select a peptide with characteristics of conformational epitopes on Mut-II.

Materials and Methods

Animals and venoms

Eight-to-nine-week-old female New Zealand white rabbits (2.0-2.5 kg) were maintained at the Centro de Bioterismo, ICB-UFMG (Belo Horizonte, MG, Brazil), and received water and food under controlled environmental conditions. Treatment and handling of all animals used in the experiments followed the requirements of the Ethics Committee

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of Animal Experimentation (CETEA) of UFMG. *L. muta muta* venom was obtained by milking specimens captured near Manaus, Amazonas, Brazil and raised at the serpentarium of Fundação Ezequiel Dias (FUNED), Belo Horizonte, Brazil. Mut-II was isolated as previously described by [12].

Molecular modeling of 3D structure of the Mut-II

Mut-II 3D structure model was constructed based on its homology to atrolysin C (a hydrolase; EC 3.4.24.42) bound to batimastat which is a potent metalloproteinase inhibitor (pdb code: 1dth.pdb) utilizing the Swiss-Model as described earlier [13]. The validation was performed by Anolea tool [14] and the visualization of the model by the DeepView/Swiss-PDB-Viewer software [15]. The predicted epitope was located in the three-dimensional model of Mut-II and visualized using Swiss-PDB-Viewer package [15].

Prediction of the epitopes by selecting amino acid residues with specific physical-chemical and structural attributes available in Blue Star Sting database

Due to the rich repository of protein characteristics offered by BlueStar STING (BSS) platform [16-19], which is the latest version of SMS, has already been used to predict enzyme class [20], protein-ligand analysis [21,22], protein mutant analysis [23,24], protein-protein interaction pattern analysis [25] and others [26,27]. In this work, BSS platform was used as a tool to predict a conformational epitope of Mut-II based on its physicochemical properties. For this, we used Java Protein Dossier (JPD) module of BSS [28] as a tool to select amino acids, which could form possible epitopes in the 3D model of the Mut-II. In the JPD, there is a SELECT feature that allows the user to filter conditions that may be used to select amino acid residues which satisfy all indicated numerical and/or descriptive ranges marked in the interactive SELECT window. In this case, our intention was to select those amino acid residues, which satisfy a very simple set of rules: being hydrophilic and possessing the area of accessibility larger than 100 square angstroms. This cascade of two filtering conditions is easily marked in JPD select feature by selecting first: "Relevant Sites" and marking Residue Location (Surface) and then, "Surface Accessibility" and marking Surface Accessibility (Surface Accessibility in Isolation >100). For hydrophilicity, one shall mark the select feature "Others" and then "Residue Property", marking "Hydrophilic" residues. By pressing OK, those two filters would be applied and only amino acid residues that satisfy both conditions would remain displayed in the JPD window. This procedure was actually the initial step for selecting residues for the final composition of the peptide. Twenty-five residues were selected.

In the last step of the epitope prediction, we proposed a peptide by combining some of these residues with its spatial neighbours. For this, using the Swiss PDB Viewer we visualized in the Mut-II 3D structure the 25 residues selected by the JPD-BSS. We saw that the Y116, Y117, Q194, L197 and K199 residues were spatially near each other (radius smaller than 10 Angstroms). Therefore, to bond these residues to each other and obtain a peptide sequence, we used their spatial neighbours to link one residue to another. The residues used as spatial neighbours were selected for being in the shortest distance from the two residues selected by the JPD-BSS. Thus, in-between Y116 and Q194 residues, we selected the C115 residue for being the spatial neighbour of these two residues. In like manner, in-between Q194 and L197 residues we selected the C195 residue, and in-between L197 and K199 residues we selected the N198 residue. It was not necessary to add a residue in-between P117 and Y116. Lastly we added the P200, Y5 and L48 residues to the end so that the surface formed on the peptide could

homogeneous. Therefore in the end, we obtained a peptide composed by discontinuous regions of the Mut-II: P117-Y116-C115-Q194-C195-L197-N198-K199-P200-Y5-L48.

Synthesis, purification and mass spectrometry of soluble peptide

The peptide PYCQCLNKPYL was synthesized in a ResPep SL/AutoSpot SL (Intavis, Germany) synthesizer by Fmoc chemistry Technique [29] as adapted by [7]. The peptide was released from the resin by trifluoroacetic acid treatment in the presence of the appropriate scavengers. The synthetic peptide (40 mM) was diluted in Milli-Q water (5 ml) and purified by high performance liquid chromatography (HPLC) on a C18 reverse phase column (flow rate 1.0 ml/min; Vydac). The peaks were submitted to MALDI-TOF-TOF analysis.

Immunogen preparation by peptide incorporation in liposomes

The soluble synthetic peptide was encapsulated in liposomes using the dehydration-rehydration method [30]. A parallel preparation of empty liposome (PBS-loaded) vesicles was also performed. The fraction of peptide entrapped in the liposomes was determined indirectly by centrifugation (43,000×g; 30 min; 4°C) and peptide titration in the supernatant.

Rabbit's immunization

New Zealand white rabbits were used for the production of anti-peptide antibodies. After collection of pre-immune sera, three rabbits received an initial subcutaneous injection of 100 µg of synthetic peptide entrapped in liposomes containing aluminum hydroxide (day 1). Nine similar booster injections were made with intervals of 14 days. The animals were bled 1 week after the last injection. As control, two rabbits were also immunized using empty liposomes, prepared as described previously.

Neutralization assays

Hemorrhagic activity was assayed as described in the Kondo method [31] and adapted by [12]. Aliquots of *L. muta muta* venom [amount equivalent to 1 minimum hemorrhagic dose (MHD)/kg] in 100 µl physiological saline, or saline alone, were injected 15 days after the last immunization, into the dorsal shaved skin of the immunized and control rabbits. Twenty-four hours later, the rabbits were euthanized and the back skin was totally removed in order to photograph and measure the hemorrhagic lesions. The MHD used throughout this study was 20 µg, and it was defined as the dose, which causes a hemorrhagic lesion of 10 mm in diameter.

Enzyme linked immunosorbent assay

Maxisorp plates (Nunc) were coated overnight at 4°C with a 10 µg/mL solution with three different antigens separately (i.e. designed peptide, mut-II or *L. muta muta* whole venom) in a Coating buffer, pH 9.6, and blocked with PBS-Tween 0.1% containing powdered milk (30 g/L). Antibody binding was detected by horseradish peroxidase conjugated goat anti-rabbit whole-IgG (Sigma Aldrich 1:10,000), followed by addition of Sigma Fast OPD tablet (Sigma). Absorbance values were determined at 492 nm with a Bio-Rad 680 microplate reader.

Western blotting

For western blotting, 20 µg of either *L. muta muta* crude venom or mut-II were subjected to SDS-PAGE (12%) in non-reducing conditions.

The proteins were transferred onto nitrocellulose membranes and blocked with PBS-Tween 0.3%. The membranes were incubated with either anti-peptide, anti-mut-II or non-immune sera separately (dilution 1:25) for one hour at room temperature. Immunoreactive proteins were detected using anti-rabbit IgG conjugated with peroxidase (1:10,000) from Sigma. After washing three times for 5 minutes with PBS-Tween 0.05%, blots were developed using DAB/chloronaphthol, according to the manufacturer's instructions.

Immunoassays with cellulose-bound peptides

Immunoassay with cellulose-bound peptides was used to evaluate the recognition of epitope-peptide by rabbit anti-peptide serum. Sets of 12 mer-peptides were prepared on cellulose membranes by Spot synthesis [32]. Identification of essential amino acids for binding antibody-peptide was also performed in an ALA-Scan assay. In this experiment a membrane possessing a set of peptides in which each amino acid in the original sequence was replaced by an alanine at a time. The membranes were obtained from Intavis (Köln, Germany) and the F-moc amino acids from Novabiochem. The immunoassay was performed as described in [33]. The anti-peptide rabbit sera were diluted 1:1000 and alkaline-phosphatase conjugated anti-rabbit antibody (Sigma) was diluted 1:3000.

Results

Molecular modeller and Identification of conformational epitopes

We used the website BSS platform to predict/select those amino acid residues that could constitute a potential conformational epitope. The BSS itself is not a program for predicting epitopes; it is a web based software for interactive analysis of the sequence/structure/function of proteins and their complexes with DNA and ligands, allowing the user to interactively analyze molecular structures, cross-referencing visualized information with correlated attributes stored in the BSS database [11]. BSS has a module, (the JPD) [28] that communicate a large set of the physicochemical properties of proteins at a residue-by-residue level through a unique graphic interface. JPD currently connects to a total of 1540-numerical/textual descriptors for any given protein structure deposited in the PDB in a residue by residue manner ($(8.89 \times 10^{10}$ records -105000 structures with 2.2 chains in average, with 250 amino acids in average per chain) [10]. We adapted the JPD to select the potential amino acid residues with epitopic function. For this, we used the JPD to select amino acid residues that are both hydrophilic and also have high accessibility in Mut-II. Twenty-five residues were selected by the combination of these parameters, they are: K19, N21, N25, N36, N59, Q60, N64, N70, K74, N86, S89, N104, Y116, P129, K149, N153, S158, P172, Q185, M186, K190, K192, Q194, L197, K199 and can be visualized in the 3D structure of Mut-II (Figure 1A and Table 1). The conformational nature of the epitope assemblage is evident since the selected residues are not found in a linear sequence in the primary structure. The residues were grouped into two clusters, according to the spatial distance in the 3D structure. The group containing the highest number of selected residues had a peptide designed. The 3D peptide structure was manually designed in SPDBV tool combining the selected amino acid residues with their spatial neighbours to obtain a peptide with physical-chemical characteristic similar to that of the potential epitopic region of the Mut-II. Thus, the cluster possessing the residues: Y116, P117, Q194,

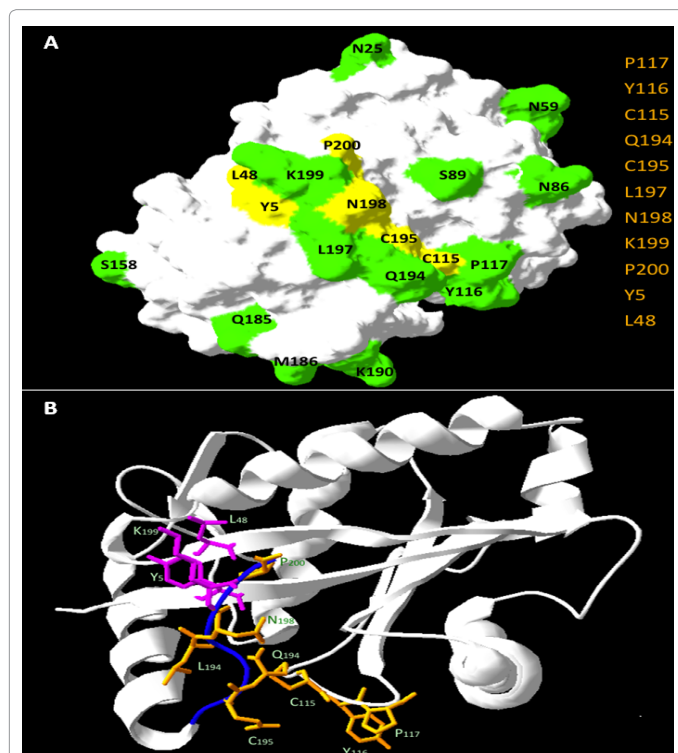


Figure 1: Visualization of the residues selected by STING as epitopes in the Mut-II and a peptide proposed in a 3D structure. In (A): visualization of amino acid residues on the molecular surface of the Mut-II. The amino acid residues selected by STING are in green and the neighbor amino acid residues used to propose the peptide are in yellow. (B): Location of the peptide in the predicted 3D structure of Mut-II. In blue, the C-terminal region; in orange, the amino acids that compose the peptide; and in magenta, the essential amino acids identified by AlaScan.

Amino Acid	Position	Accessibility (Å ²)
K	19	109.38
N	21	142.06
N	25	104.80
N	36	103.04
N	59	127.00
Q	60	112.06
N	64	103.86
N	70	102.32
K	74	120.55
N	86	133.90
S	89	101.50
N	104	151.70
Y	116	101.80
P	117	100.90
K	149	129.29
N	153	110.05
S	158	113.30
P	172	129.92
Q	185	115.42
M	186	138.18
K	190	163.35
K	192	100.50
Q	194	166.60
L	197	101.60
K	199	170.63

Table 1: Accessibility Surface of the Selected Residues.

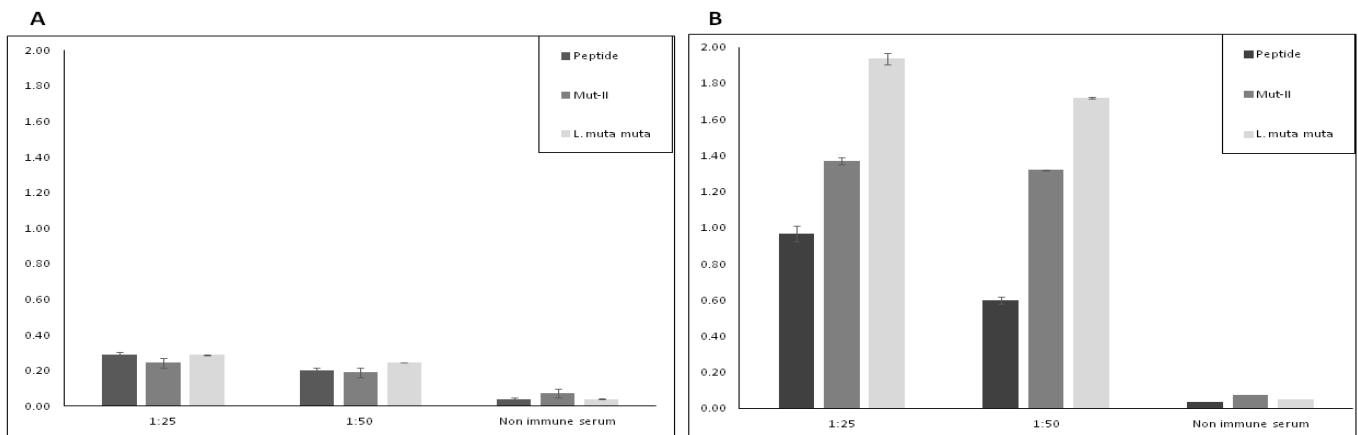


Figure 2: Immunoreactivity of anti-peptide and anti-mut-II against the designed peptide, mutalysin-II and *L. muta muta* venom by ELISA. (A) ELISA showing the reaction of anti-peptide antibodies against peptide, mut-II or *L. muta muta* whole venom. (B) Anti-mut-II was also tested against the same antigens, showing a stronger but similar pattern of bands recognition compared to anti-peptide serum. In both cases microtiter plates were coated with 10 µg/mL solution of each antigen. The absorbance of the samples was determined at 492 nm.

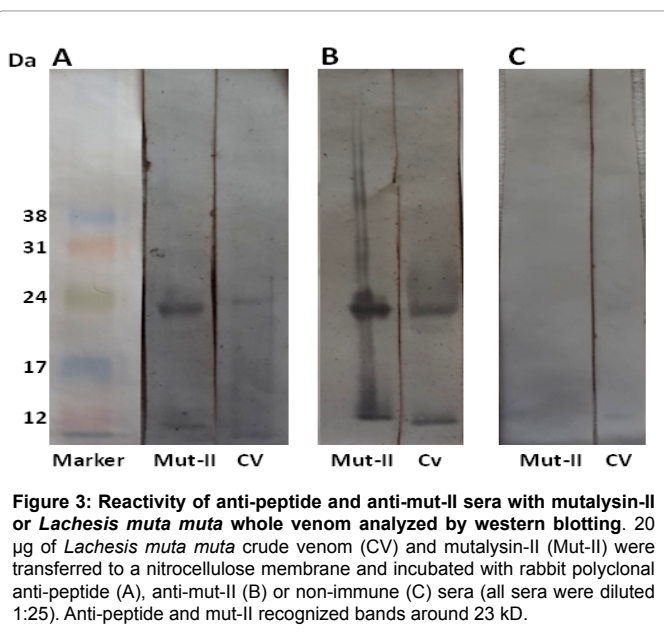


Figure 3: Reactivity of anti-peptide and anti-mut-II sera with mutalysin-II or *Lachesis muta muta* whole venom analyzed by western blotting. 20 µg of *Lachesis muta muta* crude venom (CV) and mutalysin-II (Mut-II) were transferred to a nitrocellulose membrane and incubated with rabbit polyclonal anti-peptide (A), anti-mut-II (B) or non-immune (C) sera (all sera were diluted 1:25). Anti-peptide and mut-II recognized bands around 23 kDa.

L197, K199 (Green at Figure 1A), along with its spatial neighbours, Y5, L48, C115, C195, N198, P200 (Figure 1A), were combined, to obtain the peptide P117-Y116-C115-Q194-C195-L197-N198-K199-P200-Y5-L48 (Figure 1B).

Neutralizing ability of anti-peptide antibodies

In order to confirm it as a conformational epitope, the peptide sequence PYCQCLNKPYL was synthesized and its immunogenicity tested. The peptide was synthesized using Fmoc Chemistry Technique [7,29]. The N-terminal portion was acetylated and the C-terminal portion was amidated. Subsequently, it was purified by HPLC in C18 columns and the major peak was validated by mass spectrometry, having an expected monoisotopic mass of 1382.67Da (data not shown).

After, the peptide was incorporated into liposomes using the dehydration-rehydration method [30]. Then, three New Zealand

white rabbits were immunized with liposome containing the peptide. As a control, two rabbits were also immunized with empty liposomes, containing no synthetic peptides. After the first immunization, eight boosters were performed as described before. Seven days after the last immunization the rabbits were bled and their sera were obtained by centrifugation at 3000 rpm for subsequent analysis in ELISA and Western Blotting.

Antibodies raised against the designed peptide were able to recognize both purified mut-II and *L. muta muta* crude venom in ELISA (Figure 2A) and in Western Blotting (Figure 3A) in a similar manner compared to anti-mutalysin-II serum (Figure 2B and Figure 3B). Fifteen days after the last immunization, we conducted a hemorrhagic assay to verify whether this peptide would also be capable of inducing the production of neutralizing antibodies against the hemorrhagic toxins in *L. muta muta* venom. Thus, 20µg/Kg of *L. muta muta* venom were injected intradermally into immunized rabbits and after 24 hours the rabbits were euthanized in a CO₂ chamber and their skins were removed in order to analyze the hemorrhagic lesions caused by the venom. Figure 4 shows the visceral side of the skins removed from the dorsum of the rabbits. The two rabbit controls, which received only the liposome without peptides during their immunizations, showed the greater hemorrhagic injury. In contrast, the three rabbits immunized with the peptide showed no signs of bleeding, thus indicating that immunizations were efficient for the production of neutralizing antibodies against bleeding factors in the venom of *L. muta muta*.

To verify which amino acids residues in the peptide were important in the interaction with rabbit anti-PYCQCLNKPYL polyclonal antibodies, a series of alanine analogs of the peptide was prepared by Spot synthesis and assayed for reactivity against anti-PYCQCLNKPYL antibodies. In each spotted peptide, an amino acid at a time was substituted by alanine. The results (Figure 5) indicate that in the C-terminal part of the peptide some residues could not be changed into alanine without decreasing their antibody binding capacity: this is the case for residues Lys8, Tyr10 and Leu11. These residues are, thus, key contributors in this antigenic region of the predicted conformational epitope of mutalysin-II. Residues in the N-terminal part of the peptide

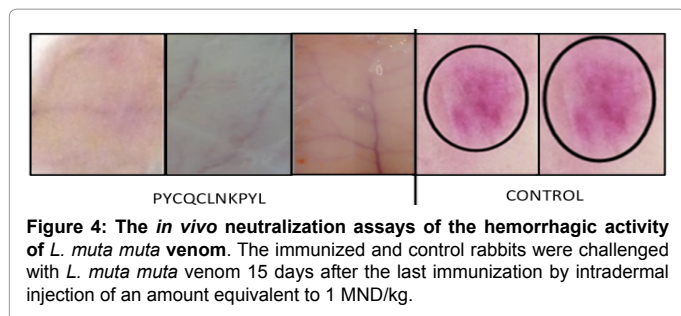


Figure 4: The *in vivo* neutralization assays of the hemorrhagic activity of *L. muta muta* venom. The immunized and control rabbits were challenged with *L. muta muta* venom 15 days after the last immunization by intradermal injection of an amount equivalent to 1 MND/kg.

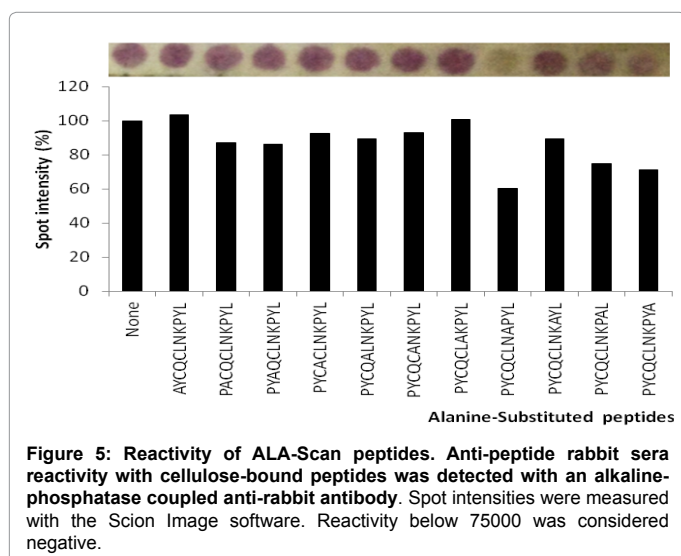


Figure 5: Reactivity of ALA-Scan peptides. Anti-peptide rabbit sera reactivity with cellulose-bound peptides was detected with an alkaline-phosphatase coupled anti-rabbit antibody. Spot intensities were measured with the Scion Image software. Reactivity below 75000 was considered negative.

play minor roles in antibody recognition.

Discussion

The peptide sequence PYCQCLNKPYL was designed *in silico* as a conformational epitope previously using BSS analysis, which selected only hydrophobic and solvent accessible amino acid residues above determined threshold. This peptide entrapped in liposomes was used to determine its immunogenicity in rabbits. Liposomes are biodegradable particles considered an excellent form to deliver biologically active substances [35]. The gradual release is essential to increase the lifetime of the peptide during its presentation to the immune system, which can improve the production of specific antibodies [36]. Anti-peptide sera produced in liposomes recognized mut-II and *L. muta muta* venom in ELISA and Western Blotting.

To measure the *in vivo* neutralization capacity of anti-peptide antibodies against the hemorrhagic activity of *L. muta muta* venom, the immunized rabbits were injected with *L. muta muta* crude venom [12,30]. Pre-existing rabbit anti-peptide antibodies neutralize efficiently the hemorrhagic activity of the crude *L. muta muta* venom [6]. Thus, it is reasonable to assume that anti-peptide serum binds to the SVMPs of the crude venom. Tanjoni et al. 2003, reported the binding of a monoclonal antibody to the disintegrin-like domain of jararhagin, a PIII-class metalloproteinase, neutralizing completely the hemorrhagic activity of the toxin. This domain is located at the C-terminal portion of the toxin. They supposed that the mechanism of this neutralization is due the proximity of this domain to the catalytic site of the protein, which probably prevented the interaction between enzyme and

substrate, thus causing neutralization [37]. Our designed peptide possesses amino acid residues located near the C-terminal region of Mut-II, as shown in Figure 1B. Despite the differences between Mut-II and jararhagin, antibodies anti-peptide of *L. muta* venom might present allosteric inhibitor mechanism. The binding of the antibody in a region spatially near the C-terminal portion of the Mut-II could cause a steric shielding of the catalytic site, blocking its binding to the substrate and thereby inhibiting the hemorrhagic effect of the toxin. The absence of K199, L48 and Y5 in analogous peptides decreases their reactivity with antibodies (identified by alanine-scanning), therefore suggesting that these amino acids are essential for the interaction with antibodies.

In this way, we suppose that the *in silico* designed peptide represents a discontinuous or conformational B-cell epitope capable to induce neutralizing antibodies against the hemorrhagic factor of the *L. muta muta* venom. Parameters such as the hydrophilicity, static accessibility and mobility of short segments of polypeptide chains have been correlated with the location of continuous epitopes in proteins [38]. B cell epitopes contain both continuous (~10%) and discontinuous (~90%) epitopes, therefore the majority of B-cell epitopes are discontinuous. Currently, only a few methods and prediction tools for discontinuous B-cell epitopes are available, and their performance is generally very poor, because they lack experimental verification [39]. Our results suggest that the accessibility and hydrophilicity are also important parameters and may be used as facilitators to predict a conformational epitope in toxic proteins. Different bioinformatics programs use specific groups of parameters, varying from one another, including in hydrophobicity, antigenicity, flexibility, accessibility, amino acids considered as special in the interactions with antibodies, segmental mobility, among others, resulting in the prediction of different epitopes, depending on the accuracy of these programs (nowadays, the programs for the prediction of epitopes have a range of accuracy varying from 35-75%) [40]. In conclusion, our results show the generation and characterization of a neutralizing conformational epitope against Mut-II from *L. muta muta* venom and that the accessibility and hydrophilicity are important parameters to predict conformational epitopes in this class of proteins.

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