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Biochemical and molecular modeling analysis of the ability of two *p*-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms

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Abstract

Snake venoms contain several trypsin-like enzymes with equivalent physicochemical characteristics and similar inhibition profiles. These are rather difficult to separate by classical purification procedures and therefore constitute a good model for affinity chromatography analysis. Some of these trypsin homologues present fibrinogenase activity, mimicking one or more features of the central mammalian coagulation enzyme, thrombin. It was previously demonstrated that a number of amidine derivatives are able to interact specifically with some of these serine proteases. To understand the enzyme–sorbent interactions we have investigated the ability of two commercially available benzamidine affinity matrices to purify thrombin-like serine proteases (TLSP) with similar biological properties from two snake venoms (*Bothrops jararacussu* and *Lachesis muta rhombeata*). Curiously, each sorbent retained a single but distinct TLSP from each venom with high yield. Molecular modeling analysis suggested that hydrophobic interactions within a specific region on the surface of these enzymes could be generated to explain this exquisite specificity. In addition, it was demonstrated that a specific tandem alignment of the two benzamidine sorbents enables the purification of three other enzymes from *B. jararacussu* venom.

Keywords: *p*-Aminobenzamidine; Thrombin-like; *Bothrops jararacussu*; *Lachesis muta rhombeata*; Affinity chromatography; Serine proteinase; Molecular modelling; Enzymatic specificity

1. Introduction

Thrombin-like serine proteases (TLSPs) isolated from snake venoms encompass a group of enzymes responsible for many important coagulation disorders in the envenomed victims [1]. These venom serine proteases are denominated "thrombin-like" because, like the mammalian enzyme, they are able to clot fibrinogen although they do not display several of the remaining thrombin activities. TLSPs have great biotechnological interest as thrombolytic agents and as diagnostic tools for coagulation disorders. The purification of these biomolecules has generally involved a combination of different steps, in particular, precipitation using salts and chromatographic procedures [2–5]. Each step has relatively low specificity and therefore affinity chromatography could be employed to circumvent this limitation since it is one of the most selective protein separation methods. Although it has only been used occasionally as an intermediary technique for isolating venom proteases, it has been extensively used on a

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large scale to remove remaining impurities of samples [6–9] and, more recently, it has been coupled to other methods to improve qualitative and quantitative proteomic recovery studies [10–12]. In addition to its technological interest, affinity chromatography is also an excellent instrument for understanding the mechanisms underlying enzyme and inhibitor interaction.

Benzamidine is a competitive inhibitor and its derivatives are known to inhibit trypsin-like proteases primarily through the interaction between the amidine moiety and an aspartate residue (D189, in trypsinogen numeration) in the enzyme's specificity pocket known as the S1 subsite [13,14]. Consequently, amidine derivatives, such as *p*-aminobenzamidine (PABA), have been prepared in different supports [15,16] and used as affinity ligands for the removal of proteases and the purification of trypsin and trypsin-like proteases such as thrombin [17,18], thrombin-like proteins [19], plasmin [20,21], urokinase [22], enterokinase [23,24], fibrinogen-clotting enzymes [25], acrosin [26,27], kallikrein [28], collagenase [29], clostripain [30] and parasite proteases [6–8].

The purpose of this study was to investigate qualitative and quantitatively the ability of two commercially available amidine derivatives resins to purify TLSPs from venoms of two important snakes widely distributed in South America. Snakes of the *Bothrops* genus are responsible for high frequency (≥90%) of snake bite events in Brazil, while the *Lachesis* genus represents the most important vipers of the Amazon region [31]. We have shown that the two analogous commercial affinity columns used in our study present distinct binding specificities and this was sufficient to identify and purify in a high yield two related TLSPs. An investigation of the possible reasons supporting this amazing specificity was carried out at the atomic level with molecular modeling. In addition, it was demonstrated that the tandem alignment of the two columns, in a definite order, allows the purification of three other different proteins from the *Bothrops jararacussu* venom.

2. Experimental

2.1. Materials

B. jararacussu and *Lachesis muta rhombeata* venoms were obtained from Institute Vital Brazil serpentarium. Centricon P10 and high performance liquid chromatography (HPLC) filters were purchased from Amicon (CA, USA) and benzamidine Sepharose 6B (Am-PABA, Lot 254128) resin from Amersham Biosciences (New Jersey, USA). *p*-Aminobenzamidine-agarose (Si-PABA, Lot 96H9534), low range molecular weight markers, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) and 4-aminobenzamidine hydrochloride were from Sigma–Aldrich Química (Brasil). Standard molecular weight markers were from Biolabs Laboratories (New England, UK). Polyvinylidene difluoride (PVDF) membrane was from Bio-Rad, sequence reagents from Shimadzu (Kyoto, Japan) and all other chemicals were of reagent grade.

2.2. Chromatographic assay

Chromatographic assays were performed on two commercial supports (Si-PABA and Am-PABA) grafted with the same amidine derivative but differing in the ligand coupling method reported by the manufacturer. Am-PABA contains *p*-aminobenzamidine covalently attached to Sepharose 6B by the epoxy coupling method and presents a spacer arm of 12 atoms [1,4,bis(2,3-epoxypropyl)butane] [32] while the Si-PABA presents the inhibitor coupled to agarose through the CNBr method and carries a spacer arm of eight atoms (glycylglycine) [33]. The same amount (500 mg) of each venom (*B. jararacussu* and *L. m. rhombeata*) was dissolved separately in water (6.0 mL), centrifuged (10,000 × *g*, 60 min, 15 °C) and the supernatant buffer exchanged against 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 M NaCl using Centricon-P10 filters. After filtration on 0.22 μm filters, a half (about 250 mg) of each venom was applied separately to each column (3 mL packed resin) or to both columns mounted in tandem (Si-PABA followed by Am-PABA). All columns were previously equilibrated with 50 mM Tris–HCl, 0.5 M NaCl, pH 7.5 and after sample application, the columns were exhaustively washed in the same buffer. Those mounted in tandem were disconnected and eluted separately. Elution of bound protein from columns was routinely carried out with 1 mM HCl, pH 3.0, containing 0.5 M NaCl at a flow rate of 1 mL min^{-1} and room temperature (25 °C). The protein content of the fractions was monitored by the absorbance change at 280 nm and the concentration estimated according to Lowry's method [34], sing bovine serum albumin as a standard.

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 12% polyacrylamide gels in Laemmli buffers [35] under reductive conditions. The gels were Comassie blue G-250 or silver stained (Bio-Rad Laboratories, CA, USA). Rabbit muscle fructose-6-phosphate kinase (84.0 kDa), bovine serum albumin (66.2 kDa), chicken egg ovalbumin (45.0 kDa) and bovine erythrocyte carbonic anhydrase (29.0 kDa) were used as molecular weight markers.

2.4. Sequence analysis

NH_2-terminal amino acid sequence of peptide samples was determined by automated Edman degradation using a gas-phase protein microsequencer (Model PSQ-1; Shimadzu, Kyoto, Japan). N-terminal sequence analysis of the protein was performed on samples electrotransferred on PVDF membranes after SDS-PAGE and treated with 5 μL of the mixture

of 0.6% tri-*n*-butylphosphine and 1.2 μL of 4-vinylpyridine in 80% isopropanol for simultaneous reduction and alkylation.

2.5. Enzymatic assays

Amidolytic activity assays were measured with BApNA as a substrate [36]. Briefly, upon addition of enzymes (1.0 μg), substrate digestion was followed by measuring the change in absorbance at 410 nm (BApNA). The substrate was digested in 50 mM Tris–HCl, pH 8.0, at 25 °C for 30 min. The activity was calculated using a molar absorption of 8800 M^{-1} cm^{-1} [37].

2.6. Inhibition of B. jararacussu TLSP by benzamidine and p-aminobenzamidine

The initial rates of enzyme-catalyzed BApNA hydrolysis were estimated from reaction progress curves at 37 °C measured by the change in absorbance at 410 nm read in a microplate spectrophotometer (SPECTRAmax PLUS^{384}, Molecular Devices, CA). Reaction mixtures (200 μL) consisting of 100 μL 2 × buffer (100 mM Tris–HCl pH 8.0) + 40 μL substrate solution + 40 μL inhibitor solution were distributed as serial substrate dilutions in 96-well plates. The later were then pre-incubated at 37 °C prior to triggering reactions by the addition of 20 μL enzyme solution containing 1 μg protein. Substrate concentrations ranged from 93 to 2500 μM BApNA whereas inhibitor concentrations ranged from 10 to 1000 μM *p*-aminobenzamidine or benzamidine. At least three inhibitor concentrations and five substrate concentrations per inhibitor concentration (in addition to the control without inhibitor) were assayed. In order to estimate inhibition constants (K_i) it was employed the "$K_{M,app}$ method" as previously described [38].

2.7. Modeling the "active" part of the immobilized ligands

Based on the docked complex between a thrombin-like enzyme from *Lachesis muta muta* (Lmm-TLE) and 4-carboethoxybenzamidine [39] we have modeled the "active" or functional portion of the ligand immobilized on the sorbents studied in this work. The "active" portion was modeled as the 4-aminobenzamidine core attached to the first four (Am-PABA) or five atoms (Si-PABA) donated by the spacer arm, in which the terminal group was truncated as a neutral methyl group. The size of the "active" portion of the affinity sorbent was selected based on: (i) the degree of similarity between the ligands/spacers immobilized on the resins; (ii) prior knowledge about the conformation of bound thrombin inhibitors and (iii) previous data obtained by our group [39]. A hundred steps of steepest descent followed by 1000 steps of conjugated gradient energy minimization were performed to optimize the geometry of the Lmm-TLE ligands, constructed as described above, already docked in a reasonable conformation at the enzyme active site. For the steepest descent procedure, only enzyme atoms at 2.0 Å distance from any atom of the ligand were allowed to move along the minimization, while enzyme atoms at 6.0 Å radius from ligand atoms were restricted to contribute for energy evaluations during the minimization, but not allowed to move. For the conjugated gradient part of the minimization schedule, these distances were extended to 6.0 and 12.0 Å, respectively.

2.8. Docking

The structures minimized as described in later section were used as inputs with optimized geometries for the program FlexX. This program is an efficient flexible docking method that uses an incremental construction algorithm to place ligands into an active site [40]. Briefly, the ligand is constructed using an incremental approach in which a base fragment (the ligand core) is automatically selected and is placed into the active site (user-defined) followed by the attachment of other remaining ligand fragments, until reconstruction of the whole molecule is completed. In particular, the conformational flexibility of the ligand is covered by generating multiple conformations for each fragment and including all in the ligand building steps. Ligands were extracted from the minimized structures and the "active-site" for ligand docking was constituted by receptor atoms 6.5 Å away from any ligand atom. None restriction was done for positioning ligands and other program parameters were default. Along with the scoring functions implemented in FlexX, functional forms included in CS-Score module were used to create a consensus score and rank the top 15 more stable configurations of ligands docked at Lmm-TLE. From these, two configurations of each ligand were chosen for the subsequent detailed study and were minimized according to the previously described protocol. In addition to a favorable score, configurations that did retain the ubiquitous salt bridge between the D189 carboxylate and the amidinium group of S1-directed inhibitors were preferred over configurations that did not. All calculations were performed using Sybyl v6.8 molecular modeling package (Tripos Inc., Illinois, USA) running on the SGI Origin 2000 server installed at the Scientific Computation Program from Oswaldo Cruz Foundation (PROCC-FIOCRUZ).

3. Results and discussion

3.1. Purification of fibrinogenases

Both *B. jararacussu* and *L. m. rhombeata* venoms are used as immunogens to obtain bothropic and lachetic horse antivenom products widely used to protect patients during an envenomation accident [31]. Serine proteases are among the venom components causing the most deleterious effects on envenomed victims. These enzymes interfere with specific points of the coagulation cascade of vertebrates, impairing blood clotting and inducing hemorrhagic disorders along with

cardiovascular failure. Therefore, in order to investigate the properties of different benzamidine derivatives to purify or remove this interesting group of proteases, the venom was fractioned using identical chromatographic conditions on two commercial supports (Si-PABA and Am-PABA) grafted with the same amidine. A schematic representation of the multiple venom proteins isolation using the two benzamidine sorbents mounted in tandem is shown in Fig. 1. As seen in the Fig. 1a the Si-PABA sorbent retained a single broad band with 48–52 kDa from the *B. jararacussu* venom (BJ-48) while no band from the *L. m. rhombeata* venom was retained (Fig. 1b). On the other hand when the *B. jararacussu* venom is fractioned first by Am-PABA, a total of four proteins (48, 32, 18 and 6–8 kDa) are retained by this column (data not shown). As expected, when the Am-PABA is connected after the Si-PABA column, it does not retain the 48–52 kDa protein but retains the three other bands from the venom of *B. jararacussu* (Fig. 1e). In addition, this same sorbent is able to adsorb a single broad band with 45–47 kDa from the venom of *L. m. rhombeata* (LMR-47, Fig. 1f). Therefore, the alignment of Si-PABA followed by Am-PABA has the advantage to purify the BJ-48 and isolate the three other proteins on the subsequent column in a single step of fractionation.

The BJ-48 presented as its N-terminal the sequence **VVGGDXIPQVPFLAF**, whereas the LMR-47 the sequence **VIGGDEXNINEHRFLVALYDGLSGT**. Both proteins are serine proteinases and had been previously characterized by us using different approaches [36,41]. LMR-47 is also probably identical to the reported 41–47 kDa SDS-PAGE migrating species that was characterized as a thrombin-like enzyme/gyroxin analogue from the venom of *L. muta muta* [42]. The 18 kDa protein (**NEDEQTRVPKEKFCLSSK-TYTKW**) was also identified as a segment from a serine protease, however the 32 kDa protein (**NNCPQDWLPM-NGLCYKIF DE**) presented high similarity with a recently identified galactose-binding protein from *B. jararacussu* venom [43]. Data listed in Table 1 demonstrate that the affinity approach used here to purify the LMR-47 protein was two times superior in recuperations and faster than that used previously by means of an isoelectrofocusing preparative method [36]. In addition, these results showed that despite both enzymes belong to the serine proteinase class and are associated with similar biological functions they present a selective binding to the two sorbents.

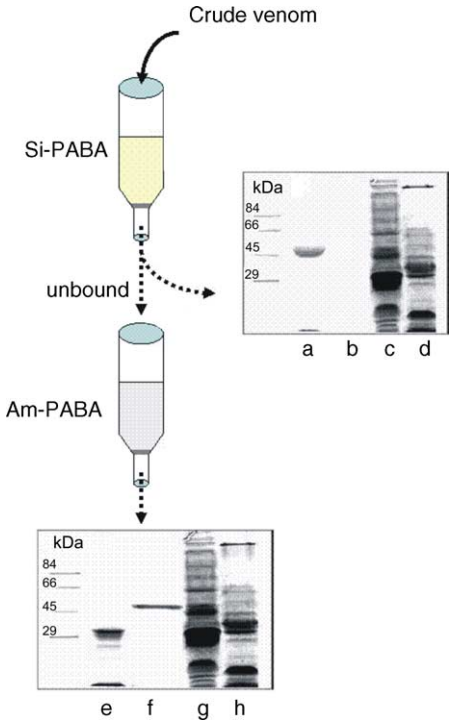

Fig. 1. Schematic representation of the multiple venom proteins isolation using the two benzamidine sorbents mounted in tandem. Venoms were applied on Si-PABA column and the non-adsorbed proteins were passed through the Am-PABA column. After exhaustively washing with the equilibrating buffer (50 mM Tris–HCl, 0.5 M NaCl, pH 7.5), the two columns were disconnected and the retained proteins eluted with 1 mM Tris–HCl buffer, pH 3.0, containing 0.5 M NaCl. The proteins were analyzed by SDS-polyacrylamide gel electrophoresis (12%). Lanes: (a) *B. jararacussu* protein retained by Si-PABA column; (b) Si-PABA column eluate; (c and g) total *B. jararacussu* venom proteins; (d and h) total *L. m. rhombeata* venom proteins; (e) *B. jararacussu* proteins retained by Am-PABA column; (f) *L. m. rhombeata protein* retained by Am-PABA column. Rabbit muscle fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa) and bovine erythrocyte carbonic anhydrase (29 kDa) were used as molecular weight markers.

3.2. Studies of sorbent binding specificity

In order to evaluate the properties of both sorbents we have investigated the effect of the ionic strength, temperature and the amino acid competitor arginine on the elution of the *B. jararacussu* 48 kDa protein from Si-PABA. Elution of BJ-48 with buffer solutions bearing increasing salt concentration is

Table 1
Purification of fibrinogenases (thrombin-like serine proteases) from *Bothrops jararacussu* (BJ-48) and *Lachesis muta rhombeata* (LMR-47) venoms

Protease	Step	Total protein		Amidolytic activity		Purification (fold)	Yield (%)
		(mg)	(%)	Total (units)	Specific[a] (units/mg)		
BJ-48	Centricon-P10	248	100	1.454	0.101	1.00	100
	Si-PABA	6.5	2.6	0.111	0.297	2.92	76.2
LMR-47	Centricon-P10	246	100	1.570	0.060	1.00	100
	Am-PABA	5.3	2.0	0.100	0.180	3.00	62.5

[a] One unity of amidolytic activity stands for 1 μmol of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrolyzed per minute, at 25 °C in 50 mM Tris–HCl, pH 8.0 buffer.

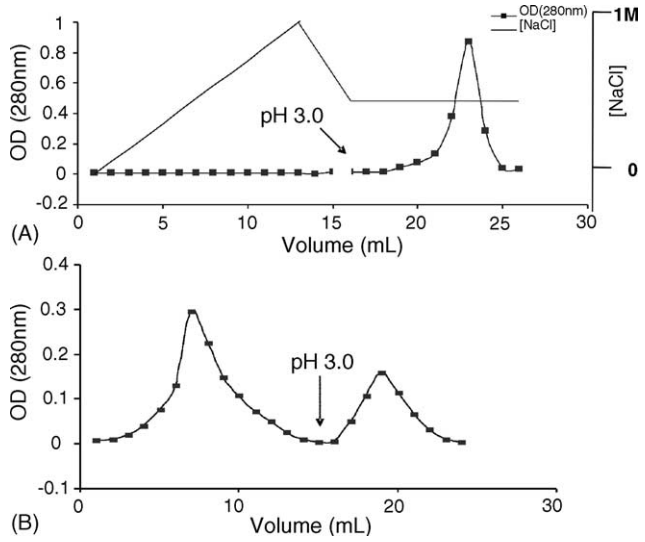

Fig. 2. Physicochemical properties of BJ-48 interaction with *p*-aminobenzamidine-agarose (Si-PABA). (A) Elution of BJ-48 adsorbed to Si-PABA resin (3 mL gel) by NaCl at various concentrations prepared in 50 mM Tris–HCl, pH 7.0, at a flow rate of 1 mL min^{-1} and 25 °C followed by 1 mM HCl, pH 3.0, containing 0.5 M NaCl. (B) Elution of BJ-48 by arginine (0.5 M) prepared in 50 mM Tris–HCl, 0.5 M NaCl, pH 7.0, at a flow rate of 1 mL min^{-1} and 25 °C followed by 1 mM HCl, pH 3.0, containing 0.5 M NaCl.

presented in Fig. 2A. BJ-48 is adsorbed at low ionic strength and cannot be desorbed by increasing the ionic strength of the eluent. This result is identical to that obtained by others [17] using bovine trypsin on dextran-coated silica supports. This observation indicates that the interaction between the proteins and the support functionalized with Si-PABA is highly specific and cannot be disrupted by high ionic strengths as would be expected if the interaction of the enzyme with the sorbent was solely due to the salt bridge between D189 and the positively charged amidine function [44].

Regarding the binding of snake proteins at different temperatures, we conducted one additional experiment at 6 °C. Neither qualitative nor quantitative difference was observed within the BJ-48 purification either if the purification experiments were developed in a cold room at 6 °C or at room temperature (25 °C, data not shown). Trypsin has been purified with *m*- or *p*-aminobenzamidine agarose at 6 °C because of the limitation of using agarose at high flow-rates and the tendency of trypsin to undergo autodegradation during purification. This is a fact not normally observed with the BJ-48 and LMR-47 proteins. As expected, BJ-48 desorption from the stationary phase took place by the competitor arginine (Fig. 2B). BJ-48 molecules adsorbed on Si-PABA were eluted with 0.5 M arginine solution, indicating that probably the same type of interactions occurred between the BJ-48 and the competing amino acid. Thus, the results described herein clearly show that the BJ-48 protein adsorb specifically to the Si-PABA since: (i) the two TLSPs (BJ-48 and LMR-47) present distinct affinity and behavior in the two sorbents and (ii) the elution of BJ-48 from Si-PABA occurs by the competitor amino acid arginine and was temperature independent.

3.3. Computer-aided evaluation of the interaction between Lmm-TLE and the functional portion of sorbents at the atomic level

Evidence has accumulated in our lab that has allowed us to safely presume that the 45–47 kDa species isolated from *L. m. rhombeata* venom using chromatography on Am-PABA resin is virtually identical to the ortologue from *L. m. muta* venom that had it primary structure determined [36,45]. In fact, the very existence of these subspecies seems not to be a consensus among herpetologists [46]. Therefore, we employed a model of Lmm-TLE previously obtained by our group [39] to study the interaction of the ligands immobilized on the resins with one of the proteins they bind specifically (LMR-47). Since both resins are claimed to be *p*-aminobenzamidine by their respective suppliers these were expected to present similar properties. Nevertheless, these sorbents presented striking distinct selectivity for the snake venoms proteins they are able to bind. One possible explanation for this observation could be based in possible differences in the affinity constants presented by the distinct enzymes for *p*-aminobenzamidine. However, inhibition data listed in Table 2 show that BJ-48 binds to this S1-directed inhibitor with comparable strength to Lmm-TLE/LMR-47. In addition, both enzymes favor, almost to the same extent, the presence of the amino group at position 4 of the benzamidine ring in order to increase binding affinity. This indicates that the S1 binding pocket of these enzymes is indeed very similar. These data encouraged us to assume that the distinct binding specificities shown by the commercial affinity sorbents based on *p*-aminobenzamidine have necessarily to be explained by the different spacer arms used to couple the *p*-aminobenzamidine moieties to the inert polymeric support (Fig. 3). Hence, we have isolated and constructed a model for the putative "active" part of the Si-PABA and Am-PABA resins and examined their most probable binding mode to Lmm-TLE.

Analysis of the top 15 configurations furnished by FlexX for each ligand docked on Lmm-TLE suggested that these could be gathered into two groups. For the ligand representing

Table 2
Comparison of inhibition constants (K_i) of S1-directed inhibitors of thrombin-like serine proteases (TLSPs) from *Bothrops jararacussu* (BJ-48) and *Lachesis muta muta* (Lmm-TLE) venoms

S1-directed inhibitor	K_i (μM)	
	BJ-48[a]	Lmm-TLE[b]
Benzamidine	110.8 ± 0.3	53.7 ± 0.1
p-Aminobenzamidine	17.0 ± 0.3	13.3 ± 0.4

[a] Inhibition was observed over reaction progress curves for α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) hydrolysis at 37 °C in 50 mM Tris–HCl, pH 8.0. Values shown along with standard errors.

[b] Constants at 25 °C using BapNA as substrate [45]. Values are shown as means ± standard deviations.

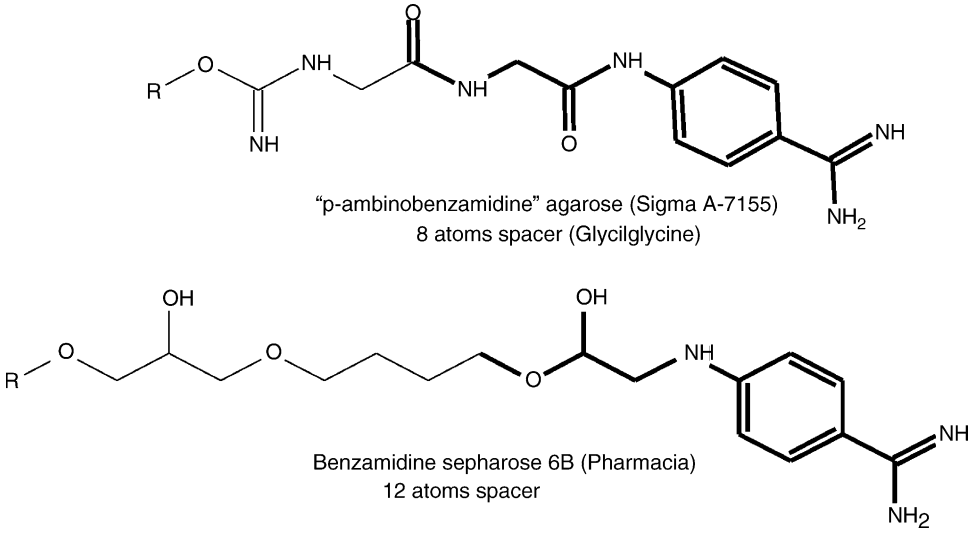

Fig. 3. Chemical structures of the *p*-aminobenzamidine ligands immobilized on commercial resins. The Si-PABA adsorbent was obtained by the manufacturing through the attachment of glycyl-glycine to the CNBr activated agarose followed by EDAC condensation with *p*-aminobenzamidine. The Am-PABA was obtained by the reaction of sepharose with 1,4 bis(2,3 epoxypropoxy/butane bis-oxirane) followed by epoxy opening with *p*-aminobenzamidine. R = cross-linked agarose.

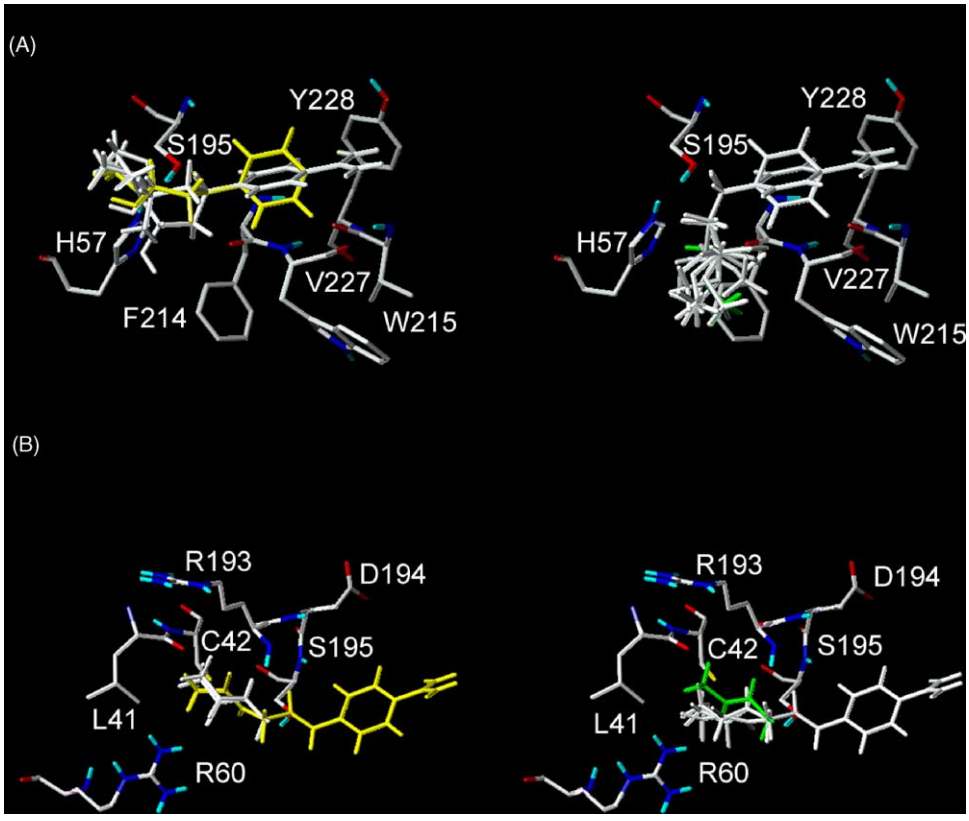

Fig. 4. Analysis of possible binding modes for the resin surrogates on the previously proposed *Lachesis muta muta* thrombin-like enzyme (Lmm-TLE) model [39]. (A) Two major groups of Am-PABA surrogate configurations: left—residues making contacts (4.5 Å radius) with 13th ranked configuration (yellow, Am-13); right—fifth ranked configuration (green, Am-5) and contacting enzyme residues. (B) Two classes of Si-PABA surrogate configurations: left—residues in contact distance from the seventh ranked configuration (yellow, Si-7); right—residues interacting with the second ranked configuration (green, Si-2). Other configurations inside each class are colored white. Enzyme residues are colored according to CPK code (oxygen: red, carbon: gray, nitrogen: blue and sulfur: yellow).

the Am-PABA "active" part, one kind of configuration, represented by the third ranked conformer was characterized by: (i) a hydrogen bond between the *p*-aminobenzamidine NH (acceptor) and OH from S195 (donor); (ii) the projection of the terminal methoxyl group to the lipophilic region composed by W215, F214, Y228 and V227; and (iii) a polar interaction between the side chain hydroxyl and H57 (Fig. 4A). The flexibility furnished by the three freely rotable bonds in the side chain from this ligand result in a number of variations of this archetypal binding mode. In one of these, an additional stabilizing hydrogen bond between the hydroxyl moiety and H57 could be attained (Fig. 4A, right panel). This later configuration (fifth in rank, Am-5) was selected for further energy minimization and detailed analysis. The second selected configuration (13th in rank, Am-13) differs from the first in that the directionality of the hydrogen bond between the NH and S195 was inverted as well as methoxyl points to the S1′ subsite and makes a hydrogen bond with H57 while the hydroxyl points to the solvent (Fig. 4A, left panel). In the case of the ligand mimicking the Si-PABA sorbent, the glycyl-glycine side chain brought by the spacer arm makes a beta-sheet with the polypeptide segment from residues 192 through 194, as exemplified by the second ranked configuration (Si-2). The first carbonyl (counting from the benzamidine ring) makes a hydrogen bond with the backbones of R193 and D194, in addition to the OH from S195 (Fig. 4B, right panel). The second carbonyl makes a hydrogen bond with R60, which delimits the S1′ subsite walls, while the terminal methyl points to the hydrophobic S1′ floor, composed by L41 and C42. A variant of this configuration (seventh in rank, Si-7), in which the second carbonyl is inverted, makes a hydrogen bond with NH of R193 backbone (Fig. 4B, left panel).

We decided to inspect in detail at least two configurations of each resin surrogate because we felt that a premature decision about which binding mode would be more favorable could be misleading. Analysis of the final minimized configurations of the Am-PABA resin surrogate showed that both would represent equally acceptable binding modes for this ligand. Am-5 differs from Am-13 mainly in the fact that the methoxyl group in the former is oriented to S2/S3 subsites making additional van der Waals (vdW) contacts with W99 and R174, whereas this moiety in Am-13 is facing the S1′ subsite, promoting two hydrogen bonds with the OH from

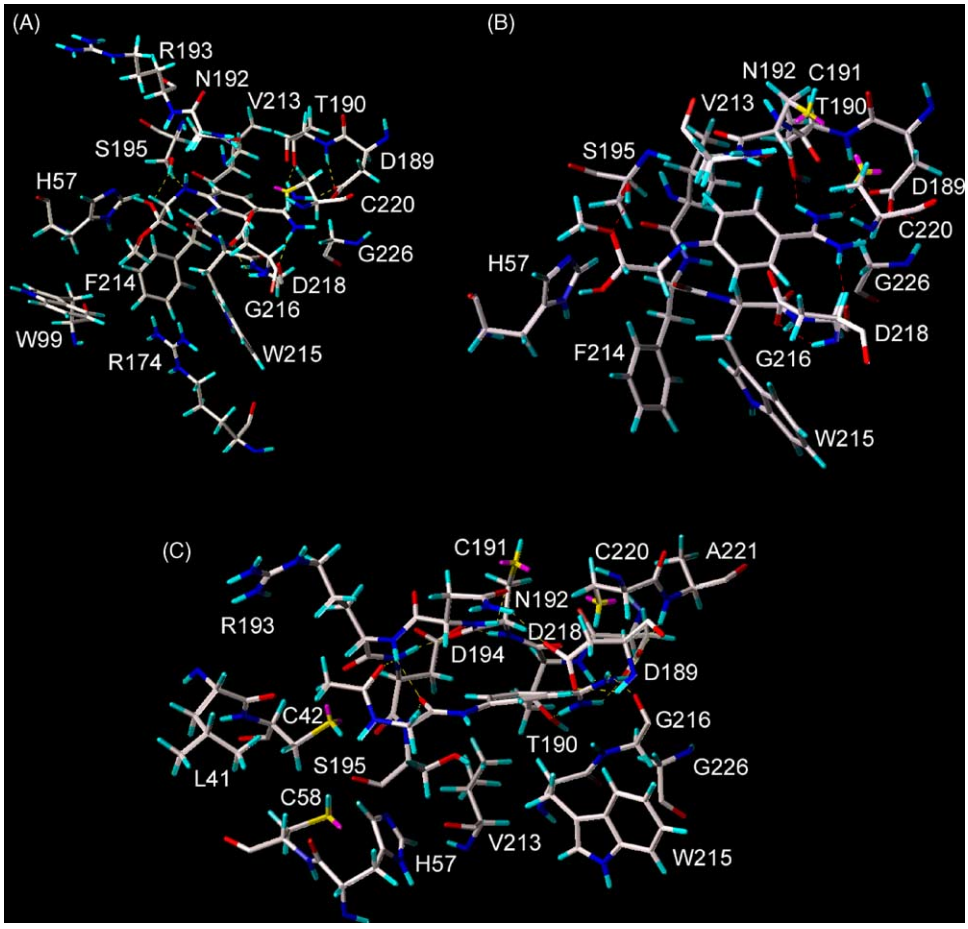

Fig. 5. Analysis of the minimized structures for the complexes between *Lachesis muta muta* thrombin-like enzyme (Lmm-TLE) and the resin surrogates on their most likely binding modes: (A) Am-5; (B) Am-13 and (C) Si-7. Enzyme residues and ligands colored according to CPK code (oxygen: red, carbon: gray, nitrogen: blue and sulfur: yellow).

S195, one through methoxyl oxygen as an acceptor and the other through the adjoining OH as a donor (Fig. 5A). This moiety in Am-13 is oriented to H57, but makes no hydrogen bond since the appropriate geometry cannot be achieved due to steric restrictions (Fig. 5B). Am-13 also makes vdW contacts with the R193 backbone atoms, while Am-5 does so with C191 backbone. Although the amidinium group is fixed in the face to face salt bridge with D189 carboxylate, the aromatic ring of one ligand is torsioned near 30° in relation to the other. The two studied configurations of the Si-PABA surrogate are almost identical, differing only in the ϕ dihedral of glycyl-glycine. In Si-7 the terminal carbonyl makes a hydrogen bond with NH from R193 backbone (Fig. 5C). Si-2 makes additional contacts with the guanidine group of R60 and the F214 backbone. Due to the better beta-sheet that can be accommodated by the Si-7 configuration we felt comfortable to propose that this configuration would represent the most probable binding mode for the Si-PABA surrogate.

A comparison between the binding mode of the Si-PABA surrogate ligand and the two possible binding modes for Am-PABA (Am-5 and Am-13) turns possible to speculate about why should LMR-47/Lmm-TLE bind to the later resin and not to the former. Si-PABA makes exclusive vdW interactions with L41 and C42 in S1′, C58 in S3, A221 and D194 while Am-5 makes exclusive contacts with R174 guanidinium group, W99 side chain, F214 backbone and R193 backbone (Fig. 6). The Si-PABA surrogate ligand is able to make a total of three hydrogen bonds in addition to the bonds made by the amidinium moiety. Am-5 makes two hydrogen bonds and Am-13 makes only one. Since we have showed that LMR-47 is able to bind only to Am-PABA, none of these interactions can be invoked to explain the observed selectivity. Equivalently, the total contact area of these ligands cannot be summoned to explain the observed chromatographic behavior since Si-PABA has 380 Å^2 compared to

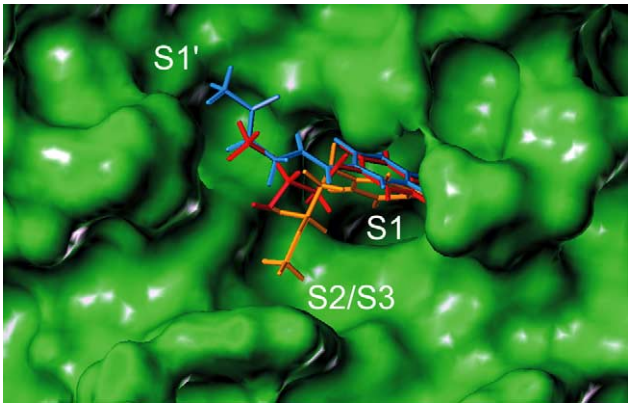

Fig. 6. Superposition of the putative binding modes of Am-PABA and Si-PABA surrogates over the *Lachesis muta muta* thrombin-like enzyme (Lmm-TLE) molecular surface. Ligands are shown as sticks (Am-5: orange, Am-13: red and Si-7: cyan). Enzyme surface (green) was calculated using a 1.4 Å probe radius. Location of specificity pockets known as S1, S1′, S2 and S3 are indicated.

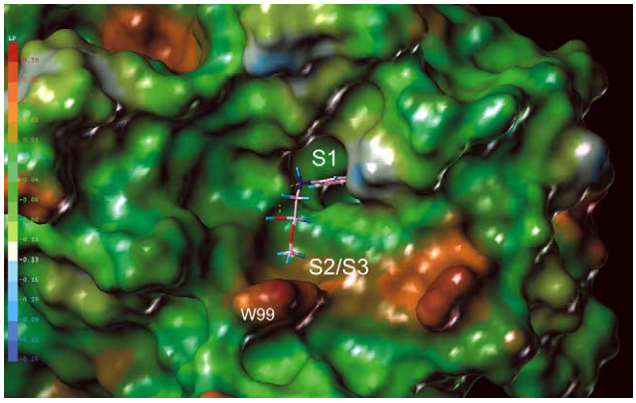

Fig. 7. Lipophilic potential of the *Lachesis muta muta* thrombin-like enzyme (Lmm-TLE) molecular surface in complex with the most likely binding-mode for the Am-PABA surrogate. High lipophilic areas are shaded in brown while high hydrophilic regions are shown in bluish tones (a lipophilicity scale is shown at left). Ligand is shown as CPK colored sticks with hydrogen bonds represented as dashed lines. The enzyme residue responsible for the high lipophilicity of the pocket receiving the methoxyl moiety of the ligand is detached.

361–367 Å^2 for the Am-PABA surrogate in the two possible conformations. The answer to this apparent contradiction must come from an unequal contribution of one of the interactions made by these ligands. The most peculiar contacts of the three analyzed complexes is the hydrophobic interaction of Am-PABA with the S3/S2 lipophilic patch, as can be observed in the binding mode represented by Am-5 configuration (Fig. 7). The importance of this interaction in defining the striking preference of LMR-47 for Am-PABA is in accordance to results recently obtained by us, which demonstrate the importance of the lipophilic potential to achieve high binding affinity to the S1 subsite of trypsin-like serine proteases [39].

3.4. Coupling of the two derivative columns to purify specific proteins

Our results indicated that the two columns possess distinct adsorptive capacity of venom proteins in spite of bovine trypsin and human plasmin being similarly retained by both columns (data not shown). Thus, in order to improve the purification process of these thrombin-like serine proteinases, the two columns were combined in tandem (using the same equilibrating buffer) as showed in the Fig. 1 (Si-PABA followed by Am-PABA). Using this procedure the BJ-48 kDa protein was obtained in high yield (2% of all venom proteins) in the first column (Si-PABA), while the second flow-through column was devoid of this enzyme and contained the 32 and 18 kDa proteins. This approach facilitated the fractionation of these additional proteins by the second column (Am-PABA) which could be easily purified to homogeneity using a further step of anion exchange high performance liquid chromatography [data not shown]. The experiments carried out with the *Lachesis* venom demonstrated the specificity of the sorbents.

4. Conclusion

We have demonstrated that similar commercial amidine derivatives supports display a strict selectivity and this could be used to purify serine proteases from snake venom with similar biological properties, in particular the BJ-48 from *B. jararacussu* and the LMR-47 from *L. m. rhombeata* venom. This specificity is probably due to the distinct chemical structures of the PABA spacer from the different sorbents, which contribute with dissimilar hydrophobic contacts to substrate binding clefts of the enzymes. The later translates into the particular chromatographic behavior observed, where BJ-48 elutes as single band from the Si-PABA sorbent while LMR-47 is exclusively adsorbed by the Am-PABA sorbent. Thus, individually the columns promote the purification of both proteins in a high yield and when aligned in tandem the isolation of other three proteins from *B. jararacussu* venom is possible.

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