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“Structural studies of PLA$_2$-like toxins and development of the structure solution method SEQUENCE SLIDER”

RAFAEL JUNQUEIRA BORGES

Botucatu – SP
January / 2017

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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Instituto de Biología Molecular de Barcelona

Departamento de Biología Estructural

PARC CIENTIFIC DE BARCELONA

Crystallographic Methods Laboratory

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (GENÉTICA)

PROGRAMA DE DOCTORAT EM BIOTECNOLOGIA

“Structural studies of PLA_2-like toxins and development of the structure solution method SEQUENCE SLIDER”

Doctoral thesis presented by Rafael Junqueira Borges, Bachelor degree in Biomedical Sciences, Ms.C. in Genetics, for the degree of Doctor (Ph.D.) from the University of State of Sao Paulo and the University of Barcelona.

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Botucatu – SP
January / 2017

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Borges, Rafael Junqueira.
Structural studies of PLA_2-like toxins and development of the structure solution method SEQUENCE SLIDER / Rafael Junqueira Borges. - Botucatu, 2017

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Marcos Roberto de Mattos Fontes
Coorientador: Isabel Usón Finkenzeller
Capes: 20901003

1. Cobra venenosa - Veneno. 2. Fosfolipases. 3. Cristalografia. 4. Biologia molecular.

Palavras-chave: Crystallography; Methods development; PLA2-like proteins; Phasing; Snake venom.

To all those who fight to eradicate human ignorance and suffering.

Acknowledgements

I express my sincere acknowledgements to the institutions and people that contributed for the development of my thesis.

Dr. Marcos Fontes, for the incentive and teachings since my early undergraduate research and for the given background that allowed me to adventure over new scientific fields and laboratories

Dr. Isabel Usón, for the innumerous scientific discussions personally and via video-conference, for the patience, persistence and dedication to introduce me to phasing and programming, and for trusting my work.

Dr. Ney Lemke, for guiding me over mathematics, normal model analysis and scientific ideas with metaphoric examples.

Dr. Mario Oliveira, for the aid collecting SAXS experiments and interpreting results.

Dr. George Sheldrick, for the hospitality receiving me in his laboratory in University of Goettingen and for being always available to answer any complicated question from chemistry to applied crystallography with an astonishing simplicity.

Dr. Randy Read, who received me in his laboratory in University of Cambridge, for the impressive scientific clear thoughts and for the assertive suggestions over my ideas.

Henrique Barcellos Campanelli, for the help in the experiments and for the opportunity to allow me to teach what I have acquired over my scientific career.

Our collaborators: Dr. Bruno Lomonte, Dr. Maria Laura Fernadez, Dr. Andreimar Soares, Dr. Luis Ponce-Soto, Dr. Sergio Marangoni, Dr. Salomón Huancahuire-Veja, for the various supplied snake venom proteins and inhibitors, and Dr. Ehmke Pohl, Dr. Isabel dos Santos, Edson José Comparetti, for the supplied crystallographic datasets.

LNLS-MX1&2 scientific instrumentation team, Dr. Ana Carolina de Mattos Zeri, Me. Alexandre Lo Bianco Dos Santos and Luciano Braga Candido, for the help setting the X-ray beamline to better meet our needs.

LNBIO mass spectrometry technicians, Dra. Bianca Alves Pauletti and Romênia Domingues, for the effort to aid sample preparation and results interpretation.

FAPESP, for the financial support during a 4-year project (Process number 2013/00873-4 and 2014/11182-5).

My colleagues in the Structural Molecular Laboratory and the Crystallographic Methods Laboratory, in special to Claudia and Massimo, for the enriching discussion and for pushing me to my best.

My colleagues in the laboratories I visited, for the effort to fasten my adaptation and for extending my scientific visit into a cultural exchange and discovery.

All my friends over the cities I lived during this sandwich PhD, Botucatu, Barcelona, Goettingen and Cambridge, for the unique experiences, relaxing moments and amazing trips.

My parents, brother and family, for the endless love, care and support.

“To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science.”

Albert Einstein

ABSTRACT

Phospholipases A_2 (PLA_2s) are one of the main component of *Bothrops* genus snakes and one of the main responsible for muscular necrosis in envenomation, a consequence not neutralized by administration of antiophidian serum. These proteins are myotoxic by disrupting membrane phospholipids by a catalytic mechanism dependent of calcium or by perturbing membrane integrity by an independent of calcium mechanism not fully known. Usually, snake toxins are purified directly from the natural source, extracted venom, and purity is a challenge due to the co-existence of various isoforms. The objectives of this thesis were to understand the non-catalytic membrane perturbation mechanism by studying the snake myotoxins and to propose a new crystallographic method to deal with impure samples, called SEQUENCE SLIDER. We performed structural X-ray crystallography studies, complemented with other biophysical techniques, such as small-angle X-ray scattering, with three bothropic myotoxins in *apo* state and complexed to natural products and inhibitors. We proposed local and global measurements to characterize and relate these states to toxin function. With the method SEQUENCE SLIDER, we aided elucidation of structures with partial sequence known by evaluating different side chain against real space correlation coefficient calculated from diffraction data. Furthermore, we developed SEQUENCE SLIDER to increase the scope of the crystallographic phasing program ARCIMBOLDO to lower resolution than the usual 2 Å. In such cases, different hypotheses of sequences of the partial traces of polyalanine are evaluated simultaneously and pushed through autotracing until the structure is phased. We improved the myotoxic mechanism comprehension and developed a structure solution method for challenging crystallographic structures.

Keywords: Structural molecular biology; Phospholipases A_2-like; Snake venom; phasing; ARCIMBOLDO.

RESUMO

As fosfolipases A_2 (PLA_2s) são um dos maiores constituintes protéicos do veneno botrópico e um dos responsáveis pela necrose muscular, consequência esta não eficazmente neutralizada pela administração do soro antiofídico. Estas proteínas são tóxicas através do rompimento ou perturbação da membrana celular em um mecanismo catalítico dependente de cálcio e outro independente, sendo este último não totalmente elucidado. Usualmente, estas toxinas são obtidas diretamente do veneno das serpentes, sendo sua purificação um desafio pela co-existência de diferentes isoformas. O objetivo desta tese foi compreender o mecanismo miotóxico independente de cálcio através de estudos estruturais e propor nova metodologia que trate de dados cristalográficos de toxinas provenientes de amostras impuras, chamada SEQUENCE SLIDER. Para tanto, cristalografia e outras técnicas biofísicas, como espalhamento de raios X a baixo ângulo, serão utilizados para estudar três miotoxinas ofídicas em estado nativo e complexado com produtos naturais e inibidores. Nós propusemos medidas locais e globais para caracterizar e relacionar a estrutura dessas toxinas a função. Com o SEQUENCE SLIDER, pudemos elucidar as estruturas de toxinas inéditas cuja sequência era parcialmente conhecida. Esta nova metodologia proposta consiste em avaliar diferentes cadeias laterais contra o coeficiente de correlação em espaço real calculado a partir dos dados cristalográficos. Em paralelo, desenvolvemos o SEQUENCE SLIDER no âmbito do método cristalográfico *ab initio* ARCIMBOLDO com objetivo de aumentar seu escopo a dados com resolução mais baixa que 2 Å. Nestes casos, diferentes hipóteses de sequências para as soluções parciais de polialanina são avaliadas mutualmente e enviadas a autotraçamento até que grande parte da estrutura seja faseada. Pudemos melhorar a compreensão do mecanismo miotóxico e desenvolver um programa que poderá auxiliar resolução de estruturas cristalográficas desafiadoras.

Palavras chave: Biologia molecular estrutural; Fosfolipases A_2 homólogas; Veneno de serpente; faseamento; ARCIMBOLDO.

RESUM

Les fosfolipases A_2 (PLA_2s) de les serps del gènere Bothrops són un dels principals responsables de la necrosi muscular en l'enverinament, una conseqüència no neutralitzada per l'administració de sèrum antiofídic. Aquestes proteïnes són miotòxiques pel seu efecte disruptiu sobre la membrana fosfolipídica, bé per un mecanisme catalític dependent de calci o bé per un mecanisme no depenent de calci poc descrit a la bibliografia. En general, les toxines de serp es purifiquen directament de la seva font natural, el verí extret, i la purificació és un repte a causa de la coexistència de diverses isoformes. Els objectius d'aquesta tesi van ser entendre el mecanisme no catalític de pertorbació de la membrana mitjançant l'estudi de miotoxines de serp i proposar un nou mètode cristal·logràfic per fer front a les mostres impures, anomenat SEQUENCE SLIDER. Es van realitzar estudis estructurals de cristal·lografia de raigs X, complementats amb altres tècniques biofísiques, com SAXS (dispersió de raigs X d'angle petit), amb tres miotoxines en estat apo i acomplexades amb productes naturals i inhibidors. Hem proposat mesuraments locals i globals per caracteritzar i relacionar aquests estats amb la funció de la toxina. Amb el mètode SEQUENCE SLIDER, hem facilitat l'elucidació de les estructures amb seqüència parcial coneguda mitjançant l'avaluació de diferents cadenes laterals contra el coeficient de correlació de l'espai real calculat a partir de dades de difracció. D'altra banda, hem desenvolupat SEQUENCE SLIDER per augmentar l'abast del programa ARCIMBOLDO amb resolució més baixa que l'habitual de 2 Å. En aquests casos s'avaluen simultàniament diferents hipòtesis de seqüències de les traces parcials de polialanina i es proven mitjançant l'autotraçat fins que l'estructura sigui resolta. Hem millorat la comprensió del mecanisme miotòxic i hem desenvolupat un mètode de solució estructural per a estructures cristal·logràfiques desafiants.

Paraules clau: Biologia molecular estructural; Fosfolipases A_2-like; Verí de serp; Phasing; ARCIMBOLDO.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASU	Asymmetric unit
Atx	Ammodytoxin
B1MMV	Structure BthTX-I complexed to MMV
BPB	4-bromophenacyl bromide
BthTX-I	First protein identified from *Bothrops jararacussu* venom
BthTX-II	Second protein identified from *Bothrops jararacussu* venom
CC	Correlation Coefficient
CC7	7 Cα centroid coordinates
CC_i	initial CC
CC_f	final CC
CC_{mc}	RSCC of main chain atoms
CCSa	RSCC calculated by *EDSTATS*
CC_{sc}	RSCC of side chain atoms
CD	Circular Dichroism
dMDiS	distance between Cβ of MDiS residues 121, and 125
iFace	interface-binding surface
LNLS	Laboratório Nacional de Luz Síncrotron
MDiS	Membrane-Disruption Site comprehended by L121, and F125
MDoS	Membrane-Docking Site comprehended by K115, R118, and K20
MMV	12-methoxy-4-methyl-voachalotine
MR	Molecular Replacement
M_w	Molecular weight
NM	Normal Mode
NSLS-I	National Synchrotron Light Source I
PDB	Protein Databank
PEG	polyethilene glycol
PCC	RSCC calculated by *PHENIX.POLDER*
PLA_2s	Phospholipases A2
PrTX-III	Third protein identified from *Bothrops pirajai* venom
R_g	radius of gyration
RSCC	Real Space Correlation Coefficient
SAXS	Small Angle X-ray Scattering
SEQSLIDER	SEQUENCE SLIDER
R_h	Hydrodynamic radius
RMSD	root-mean-square deviation
RMSF	root-mean-square fluctuation
SS	Secondary Structure
$wMPE_i$	initial weighted mean phase error
$wMPE_f$	final weighted mean phase error

Amino acid	3-letter abbreviation	1-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1 INTRODUCTION

THESIS OUTLOOK

This thesis focuses on the study of the mechanism of action of the phospholipases A_2 (PLA_2s) and PLA_2-like toxins from the snakes of Bothrops genus and on the development of the structure solution method called SEQUENCE SLIDER (SEQSLIDER).

The first chapter starts with a general introduction on the importance to study snake venom and its constituents, on ophidic accidents epidemiology and on the toxic effect of PLA_2 and PLA_2-like proteins that are still not neutralized but current antivenom. The structural information available is correlated with what is known on their toxic mechanism as a background for the discussion of our results. The methodologies that we proposed are shown as a result of this thesis and are not included in the Material and Methods.

We present results and discussions on PLA_2-like proteins in **section 2.8**. We start proposing three complementary methodologies that better characterize the more than 20 available structures. First, measuring the geometric orientation beween the two identical monomers of the dimeric PLA_2-like proteins structures using Euler Angles. Second, we measure the local variability of the monomers. And third, we calculate hydrophobic channel accessibility. We discuss the results of these three methodologies finding different toxin states related to how they damage membranes. The hypothesis raised in previous sections is tested with Normal Mode Analysis. Essential to the proposition of the previous analysis, we present the results of the complexes of BthTX-I, purified from *Bothrops jararacussu* venom, with the inhibitors zinc and MMV.

We present in **section 2.9** the experimental results of two basic PLA_2 that may resemble PLA_2-like proteins, BthTX-II and PrTX-III from *Bothrops jaracussu* and *Bothrops pirajai*, respectively. SAXS, DLS and crystallography are used to better characterize their structures. Their possible mechanism of action is discussed relating to the literature information.

Chapter 3 focuses on the second objective of this project, the proposition of a method that aids solution of crystallographic challenging datasets. A brief history of crystallography and its current challenges are described. The environment in which SEQSLIDER originated is outlined in the ARCIMBOLDO scope and in the complexity composition of venoms.

We describe SEQSLIDER focused in venoms in **section 3.7**. We describe the algorithm and how it evaluates different side chain using real space correlation coefficient. First, we test the algorithm on high resolution known structures from both natural and synthetic source. Second, we elucidate two previously unknown myotoxins with SEQSLIDER.

We describe SEQSLIDER focused in phasing within the ARCIMBOLDO scope in **section 3.8**. Again, the algorithm is first described, a known structure is elucidated, MltC, followed by study of an unknown case, FrmR E64H.

In the appendix, we attach the published articles related to this thesis. The proposition of a comprehensive myotoxic mechanism (FERNANDES et al., 2013), a review of the available structures of PLA_2-like proteins (FERNANDES et al., 2014) and the functional and structural study of BthTX-I with zinc (BORGES et al., 2017) are all related to **section 2.8**. At last, the application ARCIMBOLDO_LITE in a large set of structures (SAMMITO et al., 2015) is related to **section 3.3**.

This thesis present two symbiotic objectives, the understanding of toxins mechanism of action and the proposition of methodologies that aid solution of structures of medical importance. The structural description of PLA_2s and PLA_2-like proteins allowed the proposition of additional steps in these toxins mechanism of action. Three different bioinformatics tools are employed to evaluate both local and global structural features that may also be used to characterize structural movement from Molecular Dynamics models and other family of proteins. As a side chain evaluator, SEQSLIDER may aid structure solution of protein with unknown fold within ARCIMBOLDO scope or within sequence uncertainties.

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