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IN-SILICO AND IN-VITRO ASSESSMENT OF ENZYME STABILITY IN VARIOUS SOLVENTS USED AS STABILIZERS TO STORE SNAKE VENOM

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Abstract: Snake venom is a complex biological product which contains potentially autolytic components, and hence provides a useful tool for the study of long-term maintenance of enzymes in a competent state, both *in vivo* and *in vitro*. Therefore to simulate the stability of venom enzymes, pure enzyme controls were reconstituted in known concentration in three different stabilizing solutions namely saline (Set-I), saline+glycerol (Set-II) and PBS (Set-III). In order to understand, predict and optimize such processes, it is valuable to understand how additives such as salts, buffers and polyols e.g. glycerol affect protein stability in-silico. Currently, no methodology to foretell the effect of additives on protein conformation and stability has been established. In this work, we developed a methodology enabling the prediction of the additive-effect on the protein stability predicted in terms of RMSD from the native structure using 10ns molecular dynamic simulation. The in-silico prediction was further validated by in-vitro method using enzyme activity measurement. The in-silico and in-vitro assessment unanimously revealed saline glycerol combination as the most suitable stabilizing agent for enzymes.

Keywords: in-silico study, enzyme stabilization, Anti Snake Venom Sera (ASVS), Snake Venom, Protein stability by polyols.

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INTRODUCTION

Snake venom is highly modified saliva containing zootoxins that facilitates the immobilization and digestion of prey. It is a complex mixture of 99.5% water, while the other 0.5% consists of electrolytes, mucus, glycoprotein and enzymes [1]. Among all enzymes most commonly found in snake venom protease and phospholipase B needs special attention due to its species specificity [2, 3]. Previous studies have shown endogenous protective components active against some venom enzymes which exist naturally at the site of synthesis and keeps biological and enzymatic activities of such venoms stable over many years. However, upon secretion these secretary products are unstable and are subject to (auto) lytic degradation.

Venoms and other animal products are most often frozen immediately and lyophilized to preserve maximal activities but stability results reported till date are conflicting [4]. From these conflicting reports, it became clear that a more systematic and extensive approach to the stability of venom components is needed. Therefore, investigation of the effects of storage conditions e.g. temperature and solvent on venom components may have important implications for improving techniques used to collect and preserve venoms. These issues are of paramount importance as ideal conditions cannot be met every time everywhere. In addition, knowledge of the effects of storage conditions on enzymatic and biological activities of venoms and other natural products may reveal novel mechanisms by which these compounds are maintained in a competent but inactive state in vivo.

Proteins are never solvated by pure water in-vivo condition. Other solvent components, such as buffer salts and stabilizers, are usually added in the laboratory and in formulations of therapeutic proteins. Similarly, cytosolic milieu is crowded with many types of proteins, metabolites, nucleic acids, osmolytes, and other molecules. The presence of these “co-solvents,” generally alters protein equilibria and reaction kinetics by perturbing the chemical potential of the protein system including enzyme activity due to various strong and weak interactions. Preferential binding coefficients are rigorous thermodynamic quantities and are related to various activity coefficients and free energies via standard thermodynamic relations for multi-component solutions. Many binary mixtures of co-solvent and water are nearly ideal at low temperature and low concentration of co-solvents [5]. To be able to predict preferential binding of co-solvents and their impact on protein stability a molecular dynamic simulation is necessary [6].

Therefore in the present study, we investigated the in-vitro stability of two common enzymes found in snake venom in three different solvents stored for 120 days and enzyme activity was estimated at defined time intervals. The same milieu was simulated in-silico using molecular dynamic simulation.

MATERIALS AND METHODS

Enzyme activity study was divided into three sets. Set-I included reconstitution of enzymes in physiological saline (PS); set-II in PS+ glycerol (25% v/v) and Set-III in phosphate buffer saline (PBS). There were two enzymes (i) Caseinolytic Protease (Sigma P5147-100mg) and (ii) Group-2 is Phospholipase-B (Sigma P8914). The diluted enzyme using above solutions/stabilizers were stored in refrigerated condition (5±3ºC) till analysis. Each time the vial was taken out for analysis at defined time interval of 0, 3,7,15,30,45,60, 75, 90, 105 and 120 days. All enzyme

activity was estimated using a UV-VIS spectrophotometer. Enzyme activity was measured in triplicate and mean activity was considered for calculation. Activity at time 0 was considered to be 100% and subsequent activity were calculated as % activity as compared to time 0 as shown in activity plot for comparison.

Caseinolytic Protease Assay

Caseinolytic protease activity was assayed by a method of Eerlingen RC, et al. (1993) [7] using 5 ml reconstituted pure enzyme and casein as substrate in a total volume of 1.0 ml. This enzyme prefers to hydrolyze peptide bonds on the carboxyl side of glutamic or aspartic acid. One unit will hydrolyze casein to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per min at pH 7.5 at 37 °C (color by Folin-Ciocalteu reagent). After centrifugation, absorbance of the supernatant was read at 550 nm; activity was expressed as ΔA550nm/min/mg protein.

Phospholipase-B Assay

PLB activity was assayed by phenol, aminoantipyrine multistep spectrophotometric method of Misaki and Matsumoto (1978) [8]. The absorbance of the 0.5ml reaction mixer was read at 500 nm after 20 min of incubation at 37^{o}C. Activity of the test solution is extrapolated against the H_2O_2 standard curve, was expressed as ΔA500nm/min/mg protein.

MD simulation

The crystal structure for protease (1OP0.pdb) and PLB (1JIA.pdb) were retrieved from protein data bank (PDB). A 2 nano second (ns) MD simulation was carried out using YASARA tools using preset parameters e.g. 278K, AMBER99 force field inside a 50A° cubic cell filled with saline, PBS and 25% Glycerol in saline environment separately. Root Mean Square Deviation (RMSD) calculated from deviation of each atom before and after simulation was calculated after superposing the simulated average structure against the native energy minimized crystal structure used for simulation [9].

Result

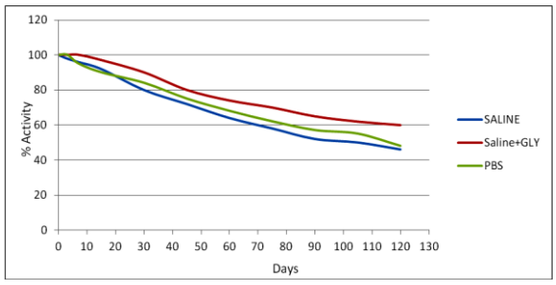
Enzyme activity at time 0 was considered to be 100% and subsequent activity were calculated as % activity as compared to time 0 as shown in activity plot for comparison in Table 1.

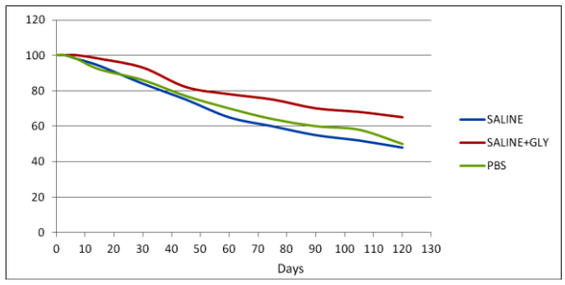
Table 1 In-vitro enzyme activity in all the 3 stabilizing solutions for both the enzymes

Days	Experimentation Result					
	SET- I (PS)		SET- II (PS+GLY)		SET- III (PBS)	
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
	Enzyme - 1	Enzyme - 2	Enzyme - 1	Enzyme - 2	Enzyme - 1	Enzyme - 2
0	100	100	100	100	100	100
3	98	100	100	100	100	100
7	96	98	100	100	95	98
15	92	94	97	98	90	92
30	80	84	90	93	84	86
45	72	75	80	82	75	77
60	64	65	74	78	68	70
75	58	60	70	75	62	64

90	52	55	65	70	57	60
105	50	52	62	68	55	58
120	48	54	60	65	52	56

Chart 1 : (a) Stability Plot for Protease (b) Stability Plot for Phospholipase-B





Both the Table-1 and Chart-1(a,b) revealed Glycerol Saline mixture as the most potent stabilizing solvent for long term storage in refrigerator without repeated freeze thaw cycle for 120 days. Group 3 and 4 showed more than 60% activity in glycerol saline mixture whereas only saline (group-1 and 2) showed less than 55% enzyme activity at the end of storage cycle. PBS showed intermediate stability (Group 5 and 6) in both the enzymes.

In-silico study involving 2ns MD simulation for both the enzymes in simulated environment revealed higher RMSD in Saline environment as compared to PBS and glycerol saline mixture as depicted in Table 2.

Table 2 RMSD values for protease and PLB in all the three solvents after 2ns MD simulation

Solvents	**Enzymes**	
	Protease (A^o)	Phospholipase-B (A^o)
Saline	2.8	2.3
Saline + Glycerol (25%)	**1.43**	**1.65**
PBS	1.85	1.92

DISCUSSION

We hypothesized that extended exposure to temperatures above freezing, particularly after dilution, would result in the degradation of enzymatic activities. Because lyophilized venom is often reconstituted, frozen and then subjected to freeze/thaw cycles as it is used, we also hypothesized that this process of freezing and thawing could damage venom components, resulting in enzymatic degradation and thus reducing the overall activities of the venom.

Native conformation, generally regarded as the most stable conformation at physiological conditions, is responsible for proper functioning of a protein. Misfolded proteins having nonnative conformations may not function properly, resulting in different physiological malfunctions [10]. Cells use two important and effective strategies to survive under stress and to reduce aggregation: the use of efficient large proteins or molecular chaperones, and accumulation of small molecules known as chemical chaperones or osmolytes.

The present study aimed at assessing the stabilizing effect of three osmolytes namely saline, glycerol saline mixture and PBS. Structural comparison between native and MD simulated structure in various environment suggested glycerol saline combination as most acceptable solvent for long term storage in refrigerated condition based on their RMSD. Both Molecular dynamics study and in-vitro enzymatic study unanimously suggested glycerol saline mixture as the most potent stabilizing solution in-vitro as compared to the rest two.

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