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Research Article
Assortment of Tachystatin B of *Tachypleus tridentatus* as a Stable Scaffold in Antifungal Peptide Design

B. Senthilkumar and R. Rajasekaran

School of Biosciences and Technology, Division of Bioinformatics, VIT University, Vellore, 632014, Tamil Nadu, India

Abstract

Background and Objective: Global increase in microbial infections has steered the production and improvement of antimicrobial and antifungal peptide drugs in pharmaceutical firm. In this study, structural stability of the antifungal peptide tachystatin B1 (2DCV) and B2 (2DCW) of *Tachypleus tridentatus* was computationally investigated. **Materials and Methods:** Static structure analysis showed high number of intra-molecular interactions in tachystatin B2 than B1 comparatively. Further, conformational sampling studies revealed tachystatin B2 to be structurally stable, substantiated by various structural events like root mean square deviation, ramachandran plot, radius of gyration, ovality and lipophilicity. **Results:** Moreover, the statistically validated contours of surface area, polar surface area, non polar surface area, hydrogen bond distribution and intra distance of disulfide bridges also supported the priority of tachystatin B2 with respect to stability. **Conclusion:** In this study, it is proposed that tachystatin B2 could be an efficient template for scaffolding antifungal peptide drugs.

Key words: *Tachypleus tridentatus*, antimicrobial peptide, tachystatin, conformational sampling, stability

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Corresponding Author: R. Rajasekaran, School of Biosciences and Technology, Division of Bioinformatics, VIT University, Vellore, 632014, Tamil Nadu, India
Tel: +91-0416-2202522

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Recent increase in the number of drug and multidrug-resistant microbial strains has possessed a great threat globally. Therefore, the need to identify innovative approaches for the development of novel anti-infectives and new therapeutic targets is of high priority in global health care. A topical survey has announced gram positive cocci as the cause for majority of nosocomial infections with *Staphylococcus aureus* (16%) and *Enterococcus* species (14%) in the lead. Additionally, invasive fungal diseases has posed a mortal threat in immunocompromised patients, as they are intricate to diagnose, treat and prevent[1,2]. Most living organisms encounter infections whose survival depends on the host defense mechanism or immunity. Vertebrates produce antibodies as part of defense naturally through innate immunity. But, immunity against infectious agents is predominantly gained through intake of antibodies, also known as acquired immunity. Anti-Microbial Peptides (AMPs) has strong antimicrobial activity against various microorganisms causing infections. Thus, AMPs have been considered as a novel class of antibiotics recently for the development of acquired immunity in vertebrates[3,4].

The tachystatins are a major defense molecule from hemolymph plasma and hemocytes of horse shoe crab. The hemolymph comprises granular hemocytes with large and small secretory granules.

These hemocytes are highly sensitive to lipopolysaccharides, which stimulates and secretes them via exocytosis[5]. Tachystatins are 41-44 amino acid peptides belonging to knottin-type family of proteins with structural and sequence similarities to neurotoxins of spider venom, in addition to plant and insect antifungal peptides. The structure of tachystatin consists of triple-stranded, anti-parallel β-sheet with a long loop connecting β-strands 1 and 2 and six cysteine residues forming three disulfide bonds enhancing its stability. Tachystatins possess an extensive spectrum of antimicrobial activity against pathogens of Gram-negative and Gram-positive bacteria along with antifungal activity too. Three hemocyte derived antimicrobial peptides are found in Japanese horse shoe crab, *Tachypleus tridentatus* as tachystatin A, B and C. Tachystatin B have two isopeptides as B1 and B2 made up of 42 amino acids. Tachystatin B1 and B2 have a high defense mechanism against fungi and gram positive bacteria than tachystatin A and C comparatively[5,6].

Tachystatin B possesses a relatively high chitin-binding property than tachystatins A and C comparatively. This functional feature aids in its antifungal property, as chitin serves as an important component in fungal cell wall and in the healing of damaged horse shoe crab exoskeleton, which is made up of chitin[5,6]. A stable tachystatin B can serve as a novel antifungal peptide with potential pharmaceutical utility[6]. The literature review showed a lack of computational studies related to structural stability of tachystatin B. Previous studies have shown the activity of the tachystatin B on various microbial and fungal samples[6]. In the present study, the stability of tachystatin B1 and B2 was computationally analyzed using conformational sampling technique. The outcome of results help to screen the best stable peptide amongst, thereby enabling the use of stable tachystatin B as a template for scaffolding future antifungal peptide drugs.

MATERIALS AND METHODS

Dataset retrieval: Protein Databank[7] was used to retrieve the structural coordinates of tachystatins B1 (2DCV) and B2 (2DCW) belonging to *Tachypleus tridentatus*. The two tachystatin namely B1 and B2 was structurally optimized and refined using Gromacs 4.6.2 software package[8] with OPLSAA force field[9]. Subsequently, the structurally minimized tachystatins were analyzed for intra molecular interactions and other parameters using conformational sampling ensembles.

Computation of Intra-molecular interactions: The energy minimized tachystatin B1 and B2 were investigated to detect the various Intra Molecular Interactions (IMIs) using Protein Interaction Calculator (PIC)[10]. The stability of a protein highly depends on the study of intra and inter molecular interactions involved[11]. Hence, the various molecular interactions like S-S bonds, hydrogen bonds, salt-bridges, hydrophobic residues, aromatic-aromatic, aromatic-sulfur and cation-π interactions exhibited in the given protein was computed using PIC program. Empirical and semi-empirical set of rules derived from standard and published data regarding the type and distance of the bond was utilized in PIC to give a numerical output[10].

Conformational sampling by t-CONCOORD: The conformational ensemble of tachystatin B1 and B2 was generated using the conformational sampling program, t-CONCOORD. The program predicted protein conformational

flexibility using geometrical consideration. Besides, the program used single input structure of a protein to generate structural ensemble with a reasonable structural constraint functionally relevant to the conformational space. Accordingly, the program involved three-step approach for generating conformers. First, the protein structure was analyzed and converted to a set of constraints involving distance, angle, chiral and planarity constraints with both upper and lower bounds. Secondly, the predefined constraints were used for rebuilding the protein structure. Starting from random atomic coordinates, the constraints were fulfilled iteratively and a new structure was constructed. Finally, this procedure was repeated several times generating an ensemble of structure. The obtained ensemble of conformational transitions served as an input for more sophisticated sampling analysis[12,13].

Computation of structural events from ensembles: The structural and residual variation in tachystatin B1 and B2 ensemble was calculated using VEGA ZZ and VMD packages. Vega ZZ, a molecular modeling package[14] was utilized for calculating Root Mean Square Deviation (RMSD), Radius of gyration (Rg), lipophilicity, ovality, Ramachandran Plot (RP), Surface Area (SA), Polar Surface Area (PSA) and S-S bond distance. The distribution of hydrogen bonds in the given ensemble was calculated using VMD[15]. The VMD is a molecular visualization program used for the display and analysis of large biomolecular structures like proteins and nucleic acids. It is a C++ language program designed with an objective that satisfies both maintenance and addition of latest features. The ensemble protein conformations obtained was analyzed with VMD to calculate the distribution of hydrogen bonds among the models of tachystatin B1 and B2[15].

Statistical analysis: The non parametric value of the various conformers obtained via t-CONCOORD was analyzed for statistical significance. Wilcoxon Signed rank test was used to analyze the two sample distribution using MS Excel. The significance of non parametric mean values of tachystatin B1 and B2 was evaluated by the computed z-score and the probability value (p-value) of Wilcoxon Signed rank test, the p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The present study evaluated various parameters that aid in the stability of tachystatin B1 and B2 of *Tachypleus tridentatus*. The PDB structural coordinate files of tachystatin B1 (2DCV) and B2 (2DCW) was retrieved and energy minimized using Gromacs for further generation of conformational ensembles. The obtained ensembles was used in dynamic analysis of various structural stability factors such as (a) Structural deviations (b) Torsion angles (c) Solvent accessible area (d) Lipophilicity (e) Hydrogen bond and (f) Distance of S-S bond length. Besides, the stability of a peptide increase with the above structural features along with the presence of S-S bonds[16,17]. Moreover, the tachystatins B1 and B2 consisted of three S-S bonds incorporated in its structure as represented[6] in Fig. 1. Initially, the various types of intra-molecular interactions exhibited by tachystatin B1 and B2 were studied.

Static intra-molecular analysis: The IMIs of the energy minimized structure of tachystatin B1 and B2 was calculated using PIC[18]. The values computed various numbers of IMIs exhibited by tachysatin B1 and B2 of *Tachypleus tridentatus.* The computed interactions shown by S-S bond, hydrophobic residues and hydrogen bonds showed an increase in IMIs of tachystatin B2 (59) than that of B1 (57) comparatively (Table 1). The results showed an increased state of interactions in tachystatin B2 related to stability, thereby prompting the study of stability features in detail.

Structural variations between tachystatin B1 and B2: Stability of a protein was validated on the basis of its exhibited structural variations[19]. The dimensional constraint of ensemble models and their consequent residues related to the variation in torsion angles were pressed as RMSD and RP[20-22]. Thus, the mean RMSD values derived from Vega ZZ showed tachystatin B2 with a reduced value 3.51Å compared to that of B1 with 3.94Å. The value suggested the presence of minimal structural deviation in the backbone stability of tachystatin B2 than that of B1 comparatively. Further, the geometrical constraint of protein

Table 1: Dataset of tachystatin B1 and B2 illustrating the various protein intra-molecular interactions (IMIs)

Tachystatin	PDB ID	Disulfide interactions	Hydrophobic interactions	Hydrogen interactions	Total no. of IMIs	Reference
Tachystatin B1	2DCV	3	8	46	57	Fujitani *et al.*[6]
Tachystatin B2	2DCW	3	7	49	59	Fujitani *et al.*[6]

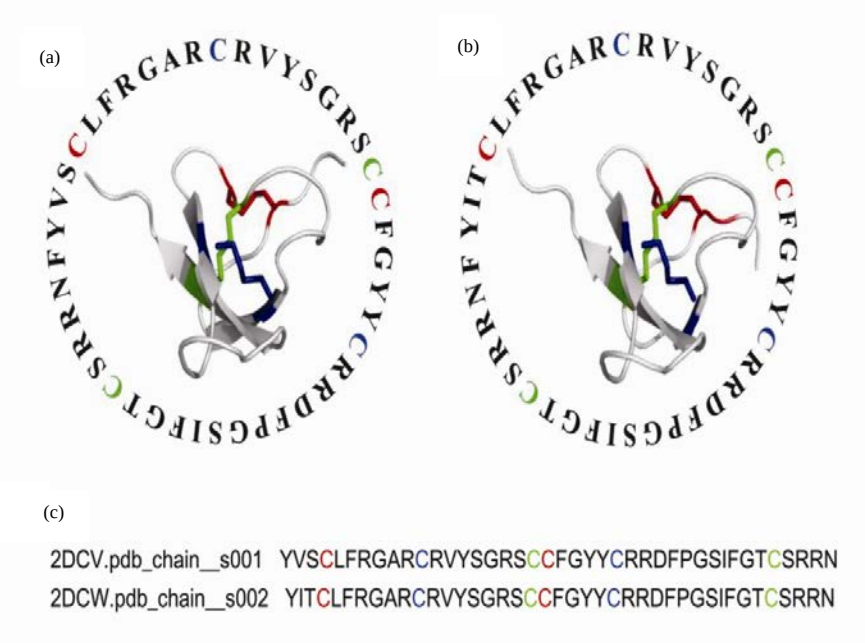

Fig. 1(a-c): Cartoon illustration of (a) Tachystatin B1 and (b) B2 of *Tachypleus tridentatus* displaying the positional constraint of three disulfide bonds (red, blue and green) along with their amino acid sequence and (c) Structure based sequence alignment of tachystatin B1 and B2 showing the conserved regions and cysteine residues

backbone was found to depend on the exhibited ψ and φ angles. The improved stability of a protein was determined by the residual distribution of torsion angles[23]. The RP has been widely used for ages to validate and appreciate the stereochemistry of polypeptide backbone[24]. Hence, the mean values of RP angular deviation in the ensemble of tachystatin B1 and B2 was calculated. Tachystatin B2 possessed a greater percentage of stereo chemical exactness of 70.72% than B1 with 69.13% (Wilcoxon Signed rank test, p-value<0.0001), where, this increase showed a improved stereochemical stability of tachystatin B2[25]. Consequently, the obtained results urged the study of structural compactness in tachystatins by computing radius of gyration.

The Rg is defined as, root mean square distance of the collection of atoms from their common center of gravity which describes the total equilibrium conformation of the given protein[26]. The compactness and three dimensional structure of a protein is exhibited as Rg and ovality[26]. The mean Rg values of tachystatin B1 was 12.19Å and B2 was 12.20Å with a minimal variation, thereby leading to the further analysis of ovality, which measured the shape of a molecule and its transformation into spherical or elliptical form Adejoro *et al.*[27]. The mean value of ovality for both tachystatin B1 and B2 showed an identical value of 4.05. Overall analysis failed to show significant variations in Rg and ovality but, the RMSD and Ramachandran plot showed a marked increase in the stability of tachystatin B2 (Fig. 2). The distribution of hydrogen bonds, another important parameter aiding stability of proteins was further studied to understand the tachystatin B stability.

Distribution of hydrogen bonds in tachystatin B1 and B2: The bond formed between a proton covalently attached to a electronegative donor and an electronegative acceptor is known as hydrogen bond[28]. Many studies on hydrogen bond have emphasized their importance in relation to protein stability and packing density[11,29]. Consequently, distribution of hydrogen bonds among the ensembles of tachystatin B1 and B2 was analyzed using VMD program, as set with parameters at a distance of 3.5 Å and angle between acceptor and donor[30] at 30°. The calculated percentage difference between the hydrogen bonds of tachystatin B2 and B1 ensembles showed a difference of 6.11% (Wilcoxon Signed rank test, p-value<0.0001) as graphically represented in Fig. 3. Thus, an increase in hydrogen bonds of tachystatin B2 might play a major role in its stability. Previous studies stated that the hydrogen bonding

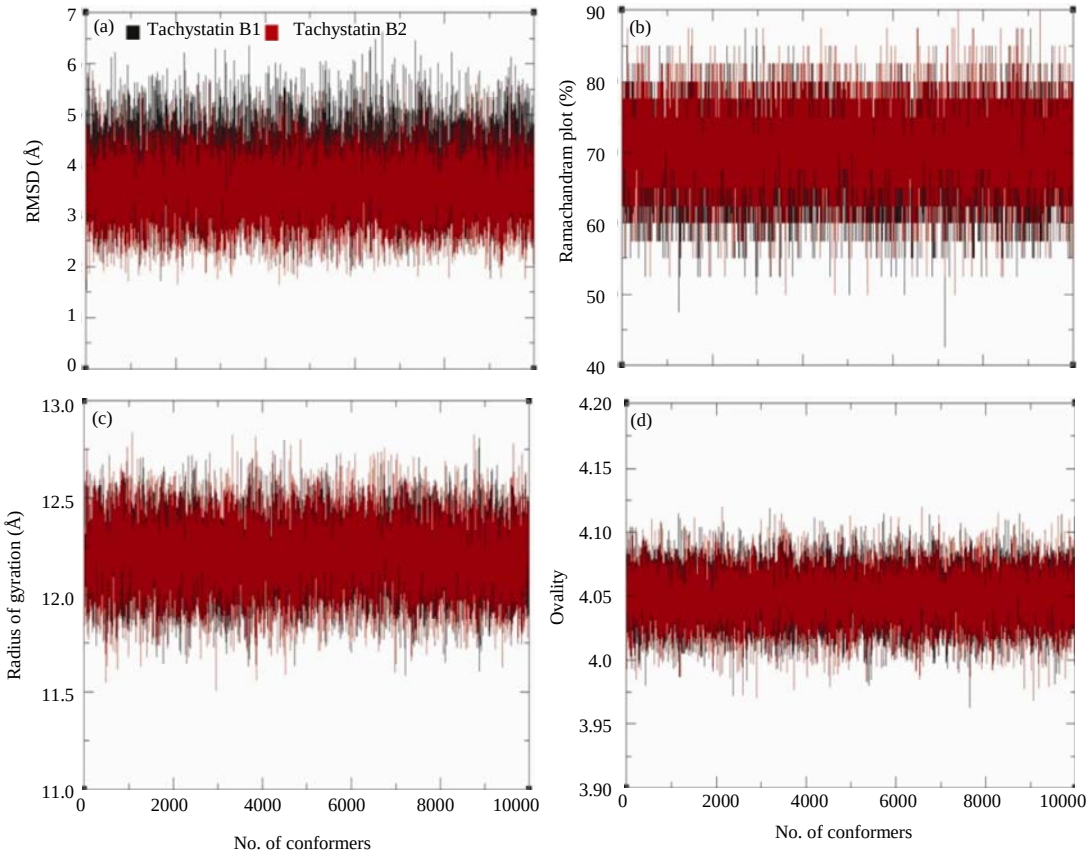

Fig. 2(a-d): Factors that decipher the structural stability of tachystatin B1 and B2 (a) Root Mean Square Deviation (RMSD) (b) Ramachandran plot (c) Radius of gyration and (d) Ovality

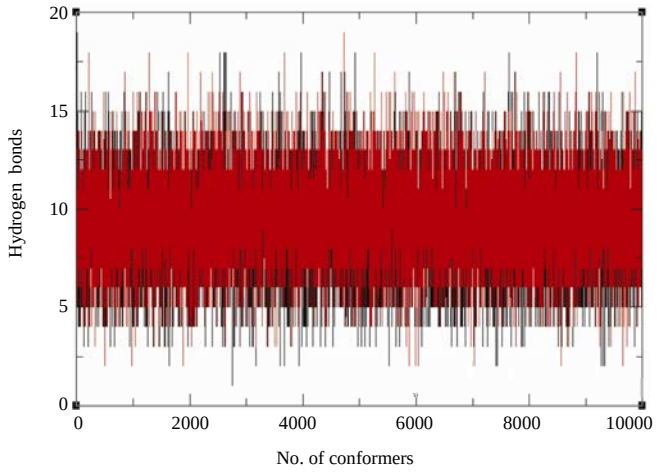

Fig. 3: Graphical distribution of Hydrogen bonds in tachystatin B1 and B2

density of water in a protein, increases along with their PSA thereby increasing its thermostability[29]. Hence, the surface area of tachystatin B1 and B2 was studied.

Solvent accessibility and Surface area of tachystatin B1 and B2: The SA, PSA and NPSA values explained the hydrophilic and hydrophobic residual features of tachystatin B1 and B2. The addition of PSA and NPSA yielded SA, which determined

surface area distribution as well as solvent accessible regions in the given peptide. The sum of surfaces constituted by polar residues (usually oxygen, nitrogen and attached hydrogen) was calculated as PSA of the given molecule. The relation between PSA and membrane permeability in peptide drug designing was explained earlier[31]. The percentage of PSA (Hydrophilicity) and NPSA (Hydrophobicity) was calculated and illustrated graphically in Fig. 4a-c. The calculated PSA percentage using Vega ZZ[32] showed tachystatin B1 and B2 with 40.8 and 40.3%, whereas that of NPSA was 59.2 and 59.7% with statistical significance (Wilcoxon Signed rank test, p-value<0.0001). An increase in the level of hydrophobicity was observed in tachystatin B2 that might aid in its structural stability. Furthermore, the apolar and polar residues constitute to functional feature of a protein, which can be analyzed by studying lipophilicity. Lipophilicity is a physicochemical parameter that aids in the determination of intake, distribution, function and removal of therapeutic drugs. Negative and low values suggested high affinity for lipids[27]. The graphical representation of molecular lipophilicity in Fig. 4d identified the mean value of tachystatin B1 (-32.13Å) to be less than tachystatin B2 (-30.52Å) comparatively. The results showed tachystatin B2 with increased affinity to lipids, thereby describing its functional weak antimicrobial activity against gram negative bacteria[27]. Additionally, the role of lipophilicity and S-S bond in functional stability of the given tachystatins was identified earlier[27,33] and therefore, S-S bond distance was studied in detail.

Distance constraint of tachystatin B among S-S bonds: Disulfide bonds improve the thermodynamic stability of a peptide by introducing conformational constraints to its polypeptide backbone. Thus, showing resistance to thermal variations, acidic or basic pH and varying concentrations of organic solvents, additionally improving half-life of peptide therapeutics[34]. Tachystatin B contains six conserved cysteine residues constituting three S-S bonds. The presence of S-S bonds increased the stability of tachystatin, thereby playing a significant role in its structural constraint[5]. Accordingly, the S-S bond distance and their distribution in the ensemble of tachystatin B1 and B2 were analyzed computationally, using Vega ZZ. The positional tier of S-S bonds in tachystatin B1 and B2 were located between C4-C20, C11-C25 and C19-C37 residues[6]. The statistically differentiated (Wilcoxon Signed rank test, p-value<0.0001) S-S bond distance of both tachystatin B1 and B2 ensemble was distributed widely between a range of 1.95 Å and 2.11 Å. On calculating the mean S-S bond distance, tachystatin B2 exhibited a closer distance ranging between 2.02 Å and 2.06 Å for larger percentage of ensemble models, while tachystatin B1 was found to exist distantly ranging between 2.02 and 2.07 Å. The three S-S bonds viz., C4-C20, C11-C25 and C19-C37 was observed to exist at mean distances 2.02, 2.02 and 2.06 Å, respectively in tachystatin B2, whereas at 2.02, 2.03 and 2.07 Å, respectively in tachystatin B1 (Table 2) with statistical significant p-value<0.0001. Though tachystatin B1 and B2 showed similar mean distance at C4-C20, tachystatin B1 failed to establish the compactness with the other two positional S-S bonds (C11-C25 and C19-C37). The former showed a decrease in compactness, whereas the latter showed an increase in structural compactness due to reduced shape and size of overall tachystatin B2 structure. Consequently, the calculated mean bond distance of S-S bonds among the ensembles of tachystatin B1 and B2 gave a broad view on their structural compactness.

Invariable studies on the distribution of S-S bond in ensembles within a defined range in a cluster form, enlightens the relation between S-S bonds and stability in depth (Table 2 and Fig. 5). Thus, the cluster S-S bond length was calculated and displayed on the basis of their conformational occurrence between the distances ranging between 1.95-1.99, 1.99-2.02, 2.02-2.05, 2.05-2.08 and 2.08-2.11 Å statistically. The clustered data forming S-S bond at C4-C20 showed high percentage of conformers as 71.97 and 10.59% at a distance ranging between 1.99-2.02 and 2.02-2.05 Å in tachystatin B2,

Table 2: Cluster distributions of conformers based on the positional constraint of S-S bridges and their bond distance variation in tachystatin B1 and B2 along with their p-values

Tachystatin with PDB IDs	Position of S-S bridges	Bond distance ranges (Å) 1.95-1.99	1.99 -2.02	2.02-2.05	2.05-2.08	2.08-2.11	Average distance (Å)	p-value
Tachystatin B1 (2DCV) Ensembles	C4-C20	81	6659	1250	968	1042	2.02	<0.0001
	C11-C25	10	5726	1795	1317	1152	2.03	<0.0001
	C19-C37	9	768	1083	1759	6380	2.07	<0.0001
Tachystatin B2 (2DCW) Ensembles	C4-C20	51	7197	1059	763	930	2.02	<0.0001
	C11-C25	6	6153	1796	1119	926	2.02	<0.0001
	C19-C37	24	1759	1452	1891	4874	2.06	<0.0001

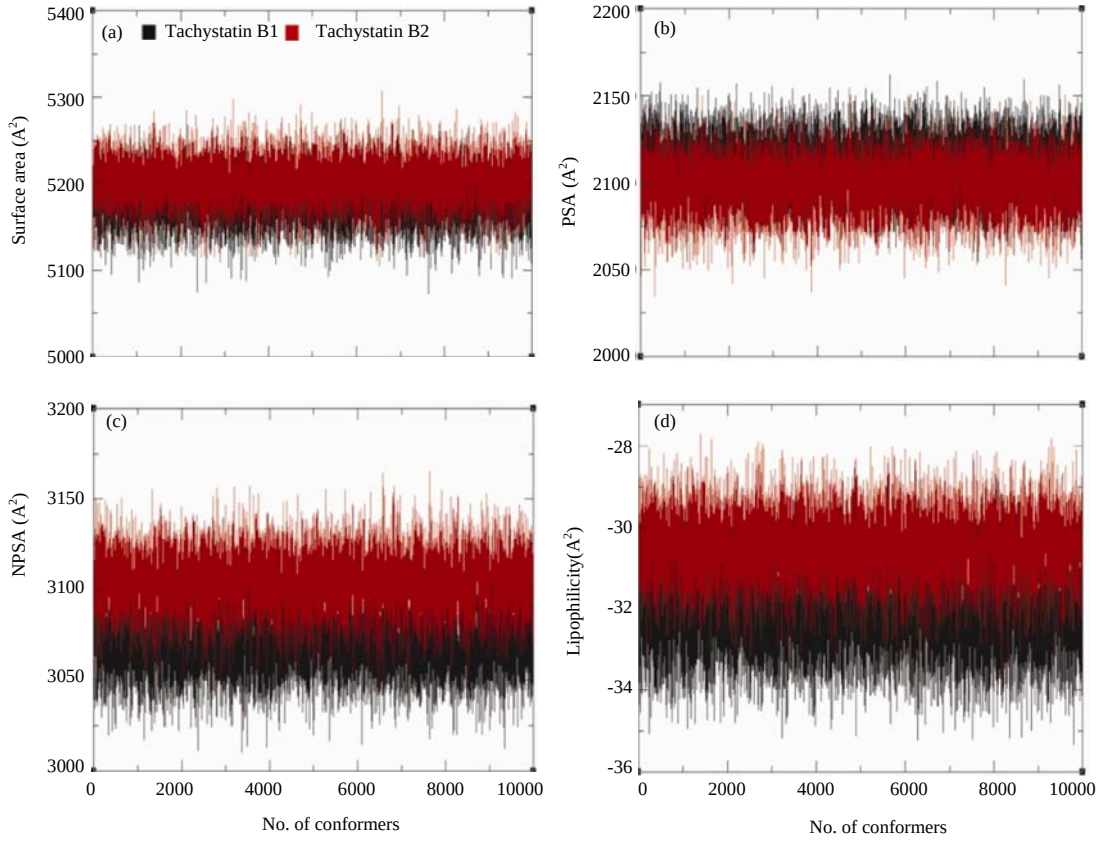

Fig. 4(a-d): Graphical representation of (a) Surface Area (SA) (b) Polar Surface Area (PSA) (c) Non Polar Surface Area (NPSA) and (d) Lipophilicity in tachystatin B1 and B2

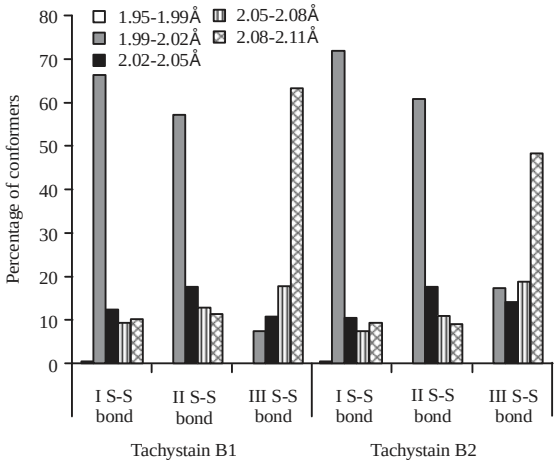

Fig. 5: Cluster distribution of tachystatin B1 and B2 conformers represented as percentage pertaining to their bond distance among the three S-S bonds

while it was found to be 66.59 and 12.50% in tachystatin B1 comparatively. Subsequently, that of C11-C25 was found to be 61.53 and 17.96% in tachystatin B2, whereas it was 57.26 and 17.95% in tachystatin B1 at similar distance range as that of C4-C20. Additionally, position C19-C37 also showed higher percentage of conformers as 17.59 and 14.52% at a distance ranging 1.99-2.02 and 2.02-2.05 Å in tachystatin B2, while it was found to be 7.68 and 10.83% in tachystatin B1. Consequently, tachystatin B1 showed 63.8% of conformers than tachystatin B2 (48.74%) at a distance ranging between 2.08-2.11 Å at position C19-C37 which caused variation in structural restraint thereby affecting stability. On contrast, the results exhibited tachystatin B2 to be more structurally stable.

CONCLUSION

Current threat of increase in fungal infection rate and its multi-drug resistance has posed a global need for the development of stable and effective antifungal drugs. Consequently, the peptide based therapeutics is under current research for the development of stable antifungal peptides. Since, cysteine rich peptides of *Tachypleus tridentatus*viz., tachystatin B1 and B2 serve functionally as antifungal peptides; we reported through computational

aspects that the static model and conformational sampling analysis favored the structural stability and functionality of tachystatin B2 than B1. Hence we conclude that tachystatin B2 serving as a stable template, could be a boon for pharmaceutical industry.

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