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In silico Characterization, Molecular Dynamics Simulation and Expression Analysis of Two Class I Chitinases from *Aloe barbadensis*

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A B S T R A C T

Chitinases are involved in chitin catabolism of fungi and bacteria but are also widely distributed in plant Kingdome as defense molecules. Chitinases are known for their diversity and vast potential uses viz., biocontrol agents for plant pathogens and insects, biopesticides, chitioligosaccharides, single cell protein production from chitinous wastes etc. Plant chitinases have been classified in various classes (Class I-VII) based on their domain organizations. In present study, two class I chitinases were identified from tropical plant *Aloe barbadensis* (family Asphodelaceae). *A. barbadensis* is well known for its diverse medicinal uses. Despite immense pharmacological uses, very little genomic and molecular information is available for this plant. In this study, two putative chitinase sequences were identified from raw transcriptome data of *Aloe barbadensis*. Their domain analysis showed that these chitinases belong to class I. Homology models were generated using putative chitinase sequences for their structural analysis. To further analyze the substrate binding for these chitinases, docking was performed with substrate. Finally, 50 ns MD simulation revealed that both putative chitinase structures are stable and shows differences in structural fluctuations. This is first report to identify such gene information from *A. barbadensis* and may be helpful in the development of potent biocontrol agents against plant pathogens.

Introduction

Plants produce diverse array of pathogenesis-related (PR) proteins to protect against attacks of external pathogens. Chitinases are PR proteins that hydrolyze chitin, a homopolymer of N-acetyl D-glucosamine. This polysaccharide is structural component of fungi, insects, crustaceans and green algae (Santos *et al.,* 2004). Plant chitinases belong to glycoside hydrolase 18 and 19 families on the basis of their catalytic mechanism. They are further classified in to seven classes (Class I-VII) based on different amino acid residues forming domains and motifs (Neuhaus *et al.,* 1996; Singh *et al.,* 2007; Ahmed *et al.,* 2012).

By contrast, the catalytic mechanism of GH19 is not wholly understood. These proteins are constitutively present in leaves, roots, stems and flowers but the high expression of their genes have been reported during external elicitations (Libantova *et al.,* 2009; Porat *et al.,* 2001; Gerhardt *et al.,* 2004). These proteins are of huge significance for their diverse nature and immense potential uses viz. biocontrol of plant pathogenic fungi and insects, biopesticides, production of chitooligosaccharides, single protein production etc. (Islam and Datta 2015). Various organisms produce a wide variety of chitinases that show biochemical as well as biophysical characteristics for different potential uses.

The genus Aloe (family Asphodelaceae) comprises around 360 species. Out of which *Aloe barbadensis Miller*, *A. arborescence*, *A. ferox* and *A. perryi* are known to exhibit medicinal properties. *A. barbadensis*, in particularly, is well known tropical succulent plant that has been known and grown for their immense pharmaceutical importance since thousands of years(Pugh *et al.,* 2001; Chen *et al.,* 2012). Bioactive constituents from *A. barbadensis* have been known for their anti-ulcer, anti-hyperglycemic, skin burn healing, anti-asthmatic, antioxidant, hepetoprotective, manti-tumer, antihypercholestrolemic, and anti-inflammatory properties (Tarameshloo *et al.,* 2012; Langmead *et al.,* 2004; Chandan *et al.,* 2007; Lin *et al.,* 2006; Borrelli and Izzo 2000; Kumar *et al.,* 2013; Huseini *et al.,* 2012; Qadir, 2009; Mikadi and Damak, 2008). Despite huge potential applications very little genomic, proteomic and gene content related information is available for *A. barbadensis*. Such limitations halt bio-prospecting at molecular or gene level of this plant. Present study is the first report on identification and computational characterization of a chitinase from *A. barbadensis*. Putative chitinase transcripts were identified in raw TSA

database. *In-silico* characterization of respective proteins of transcript sequences and their expression analysis were carried out. The sequence information of putative *A. barbadensis* chitinases were used to generate protein homology models. Further, docking with ligand and molecular dynamics simulations were carried out for *A. barbadensis* chitinase.

Materials and Methods

Identification of chitinase sequences

Chitinase [*Hordeum vulgare*: AAA56787.1] sequence was used for tBLASTn against TSA (Transcriptome Shotgun Assembly) database of *Aloe barbadensis*. Two raw sequences were retrieved based on score, query cover and length of unigenes. The ORF regions of all unigenes were determined using ExPASy tool (https://web.expasy.org/translate/). Protein sequence obtained from translate tool were again subjected to BLASTp against non-redundant database for proteins at NCBI to identify or to verify the ORF regions. Similarity percentage between identified sequences was determined by Clustal-omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). MEGA6 was used to multiple sequence alignment and phylogenetic analysis of homologs (Tamura *et al.,* 2013). For phylogenetic analysis Neighbor-joining (NJ) approach with bootstrap replicates (1000) was employed.

Gene expression analysis of chitinases in different aloe genotypes

Expression profile of Abchit genes was studied in three genotypes using Real-time PCR detection system and SYBR green PCR master mix. Gene specific primers were designed and GAPDH gene primers were designed as reported earlier (Choudhari *et al.,* 2018). Leaf samples were collected from three

different genotypes of *Aloe barbadensis* maintained at DMAPR field. Young leaves were selected to analyze the expression level of two chitinases. These Aloe genotypes have distinct morphological traits. Gel portion of Aloe leaves were removed and remaining samples were used for total RNA isolation. Total RNA from leaves was isolated using GeneJET Plant RNA Purification Mini Kit (Thermo Scientific). A first strand cDNA was prepared using 5 µg of total RNA, 500 ng oligo-(dT)18 primer (supplied with kit) and M-MuLV Reverse Transcriptase supplied with First Strand cDNA Synthesis Kit (Thermo Scientific). For each primer set, a control reaction was also included without template DNA. Real time analysis was conducted using SYBR green master mix (Maxima SYBR Green).Fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$ using ΔCt values.

Domains identification and estimation of biochemical parameters

Protein sequences were deposited at PROSITE expasy (https://prosite.expasy.org) and SMART (http://smart.embl-heidelberg.de) to identify domain structures. Protein properties such molecular weight, isoelectric point, grand average of hydropathicity were predicted using Protparam tool (https://web.expasy.org/protparam/).

Homology modeling

The protein sequences of Abchit1 and 2 were used for Blastp search tool against PDB database and four different protein crystal structures were collected based on e-values for closest homology identification. These PDB structures used as templates for model building. Modeller 9.22 was used to develop homology models of Abchit1 and 2. Above five PDB templates were used in Modeller and best template was identified based on distance matrix generated by Modeller and

crystallographic resolutions. Modeller generated 50 initial models and based on their DOPE score and GA score best three dimensional model was selected for further analysis. Models were visualized by PyMol and Discovery studio programs.

Docking analysis

A. barbadensis chitinases were docked with NAG oligomer using Autodock Vina program(Trott and Olson 2010). Structures of *A. barbadensis* chitinases and ligand were converted in to PDB format using Discovery studio. Docked protein complexes were visualized and analyzed using Discovery studio and PyMol program.

MD Simulation

A. barbadensis chitinase models were subjected to 50 ns MD simulation using GROMACS package(Berendsen *et al.,* 1995). The topology files were generated using GROMOS 96 force field. In preparative steps, The Abchit1 and 2 proteins were immersed in a box of explicit simple point charge water (SPC water). The dimension of box was kept 1 nm. To yield electrically neutral system Na or Cl ions were added. The solvated ent-CPSs were energy minimized by steepest descent methods to remove close van der Waals contacts. The system was equilibrated under NVT and NPT ensemble with position restraint simulations for 500 ps and finally production MD for 50 ns (constant temperature 300 K and pressure 1 bar) in triplicates. All the bonds were constrained using LINC algorithm and electrostatic interactions were calculated using Particle Mesh Ewald (PME). Simulations were repeated three times showing high degree of reproducibility. All trajectories like root mean square deviations (RMSD) and root mean square fluctuations (RMSF) were explored by using GROMACS. The DSSP tool was

employed to analyze secondary structure changes.

Results and Discussion

Identification of *A. barbadensis* chitinases and validation of their expression in various genotypes

Two putative chitinase sequences were identified from raw TSA database. Both sequences showed 89 percent query coverage with *Hordeum chitinase* (used as template for search) but showed 61 and 58 percent sequence identities. These chitinases were named as Abchit1 and Abchit2 which have 315 and 319 amino acid residues respectively as suggested by Expasy translation tool. Figure 1 shows the phylogenetic analysis of Abchit1 and Abchit2. Furthermore, based on Clustal-Omega analysis these two homologs show 75.56 percent sequence similarity with each other Figure 2A. It means there are subtle sequence differences between these two chitinases. Phylogenetic analysis indicated that Abchit1 is more similar to *Alium sativum* chitinase whereas Abchit2 falls in different evolutionary group (Fig. 1). Overall results showed that Abchit2 probably has evolved as distinct chitinases member. Different classes of plant chitinases show differences in domain organizations. Broadly, all plant chitinases have been classified in four classes. Class I chitinases have N-terminal leader sequence, a cysteine rich chitin binding domain, a hinge region and catalytic main domain. Class II chitinases show significant similarity with Class I chitinases but completely lack the chitin binding domain and the hinge region. Interestingly, Class III chitinases do not show significant similarity with above class I and II enzymes [25] (Collinge *et al.,* 1993). Finally, Class IV chitinases have domain organization similar to class I chitinases but the length of Class IV members is shorter (241-255 aa) than Class I members (about 300 aa). Domain

architecture of Abchit1 and Abchit2 is shown in Figure 2B. Abchit1 and Abchit2 have N-terminal leader sequence, Cysteine rich chitin binding domain (CBD) and a main catalytic domain separated by a hinge region from CBD. Figure 3 shows putative di-sulphide bond pattern formation among cysteine residues present in CBD. Based on the length of amino acid residues and domain organization, Abchit1 and Abchit2 fall in Class I of chitinases and various biochemical parameters are shown for these proteins in Table 1. Biochemical parameters show that both Abchit1 and Abchit2 have Mw around 33 kDa which is also supposed to be character of class I chitinases (Collinge *et al.,* 1993). By contrast, these chitinases have different physical nature as Abchit1 is acidic in nature whereas Abchit2 shows basic nature as indicated by their respective pI. Plant chitinases have been classified as acidic or basic chitinases (Flach *et al.,* 1992). Thus, within class-I, these chitinases can be grouped in different subclasses of chitinases. Relative expression of Abchit1 and 2 is shown in Figure 4. The results of expression analysis suggested that both of the chitinases are expressed in leaves of different genotypes. However, there are variations in the level of expression among various genotypes for individual chitinases. Our experiment shows that both Abchit1 and 2 are constituently expressed in mesophyll region of leaves.

Homology model and docking analysis of Aloe chitinases

Initially, blastp tools yielded four PDB structure (2dkv, 4dwx, 4jol and 1cns) based on homology. Finally, 2dkv (class I chitinase of *Oryza sativa* L. japonica) was selected to use it as a template for homology model for Abchit1 and 2(Kezuka *et al.,* 2010).Both chitinases show 11 helices separated by β turns and various di-sulphide bonds. Which were further validated and Ramachandaran

plot analysis of these proteins are shown in Figure 5. These analyses show almost all amino acid residues in both chitinases are under most favored region and additional allowed regions (Fig. 5 and Table 1). Both the Ramachandaran plot analysis and instability index predicted by ProtParam indicated that these three-dimensional structures are stable. Three dimensional models for Abchit1 and Abchit2 shows significant similarities.

However, the detailed catalytic mechanism and substrate binding of plant chitinases have not been elucidated yet. Although some crystal structures complex with NAG and NAG oligo have been reported (Ohnuma *et al.,* 2012; Huet *et al.,* 2008). Chitin oligosaccharides instead of NAG monomer as substrate to complex with chitinase crystal studies have appeared better way to understand substrate binding (Ohnuma *et al.,* 2012). Hence, in present study NAG oligomer was used for docking. Docking of Abchit1 and Abchit2 with N-acetyl glusamine oligomer is shown in Figure 6. AbChit1 and Abchit2 have two lobes rich in α-helical structures. Substrate binding site is located between these lobes. Figure 7 shows the amino acid residues forming contacts with substrate. Similar substrate binding was reported in chitinase from *Oryza sativum* (Kezuka *et al.,* 2010).

Various loops are extended at both end of substrate binding groove.NAG binds at substrate binding cleft similar to shown in *Oryza sativum* chitinases (Kezuka *et al.,* 2010). Also, the structural fluctuations of loop regions have shown their participation in catalytic mechanisms of chitinases. These are the structural features of G19 chitinases found in plants (Ohnuma *et al.,* 2012).

MD simulation

Three dimensional models of Abchit1 and Abchit2were analyzed by 50 ns MD simulation. Figure 8A shows RMSD analysis for Abchit1 and Abchit2. After initial increase RMSD for both chitinases achieved a stationary phase after 5 ns of simulation. RMSD of Abchit2 show some increase after 12 ns of simulation but on later stages of simulations both of proteins showed steady phase as indicated by RMSD values indicating stability of modeled proteins. Figure 8B shows the radius of gyration (Rg) of chitinases during MD simulation. This radius of gyration is an indication of compactness of proteins. Rg analysis of Abchit1showed steady states after 20 ns indicating that protein structure are compact having stable folding structure. Whereas, Rg of Abchit2 showed initial steady state up to 25 ns and then a small decrease up to 30 ns followed by steady state. Overall Rg analysis suggested that Abchit2 showed some conformational changes during simulation but Abchit1 showed comparatively stable conformation. Further, RMSF analysis will help to understand this fluctuation over MD simulation. Figure 9 showed comparative fluctuations of amino acid residues of both chitinases. RMSF analysis showed significant overlapping in comparative RMSF of both chitinases. But, Abchit2 showed more conformational fluctuations as compared to Abchit1.Overall, the main helical content showed less fluctuations and remain stable during entire simulation. Figure 10A and 10B show secondary structure analysis of Abchit1 and Abchit2 during MD simulation. These analyses indicated that over simulation time overall secondary structure of both chitinases remained unaltered.

Table.1 Biochemical parameters and Ramachandran plot analysis of Abchit1 and Abchit2

	Abchit1	Abchit2
Amino acids	315	319
Molecular weight (kDa)	32.72	33.58
pI	5.03	7.40
Instability Index	37.94	34.78
Aliphatic Index	58.98	62.82
Grand average of hydropathicity (GRAVY)	-0.191	-0.202
Ramchandran Plot analysis Residues in most favored region	86.4%	86.7%
Residues in additional allowed region	12.6%	12.8%

Fig.1 Phylogenetic analysis of various chitinases. Phylogenetic tree was generated using MEGA6 prog
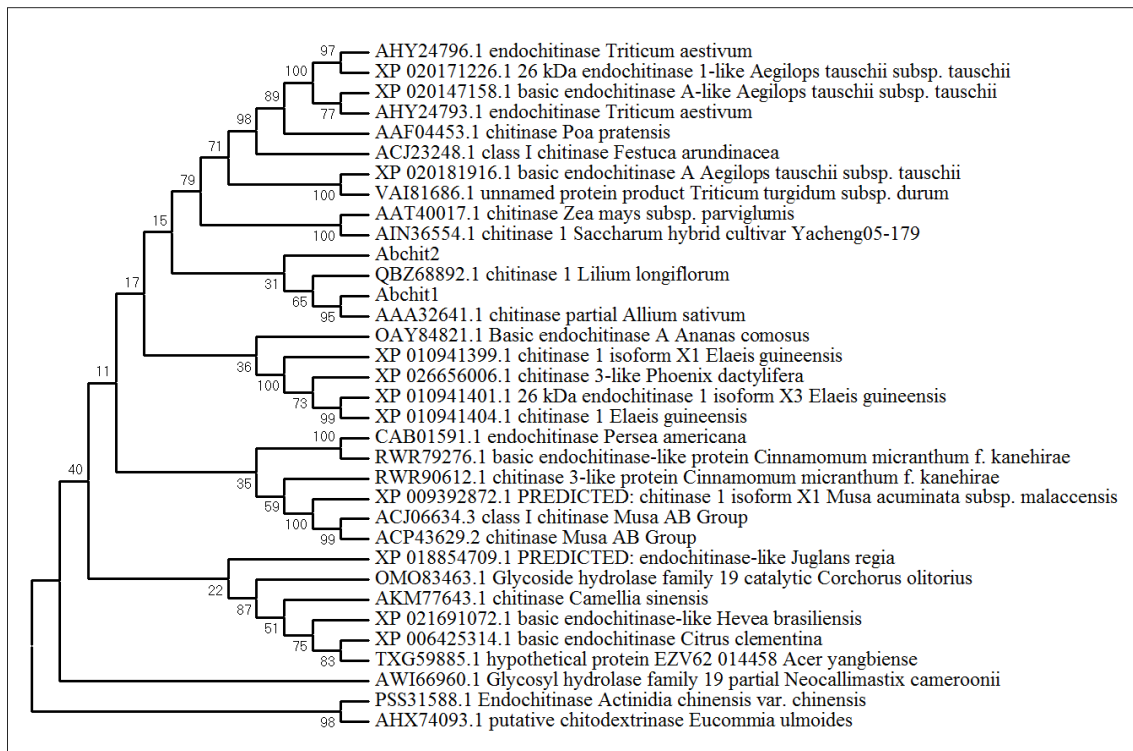
Fig.2 Sequence alignment and domain organization of Abchit1 and Abchit2. A. Clustal-omega based sequence alignment of identified chitinases. B. Domain identification and their arrangement in Abchit1 and Abchit2 based on SMART and Prosite analysis. N- to C- terminal: Leader sequence, Cysteine rich Chitin Binding Domain (CBD) and main catalytic domain
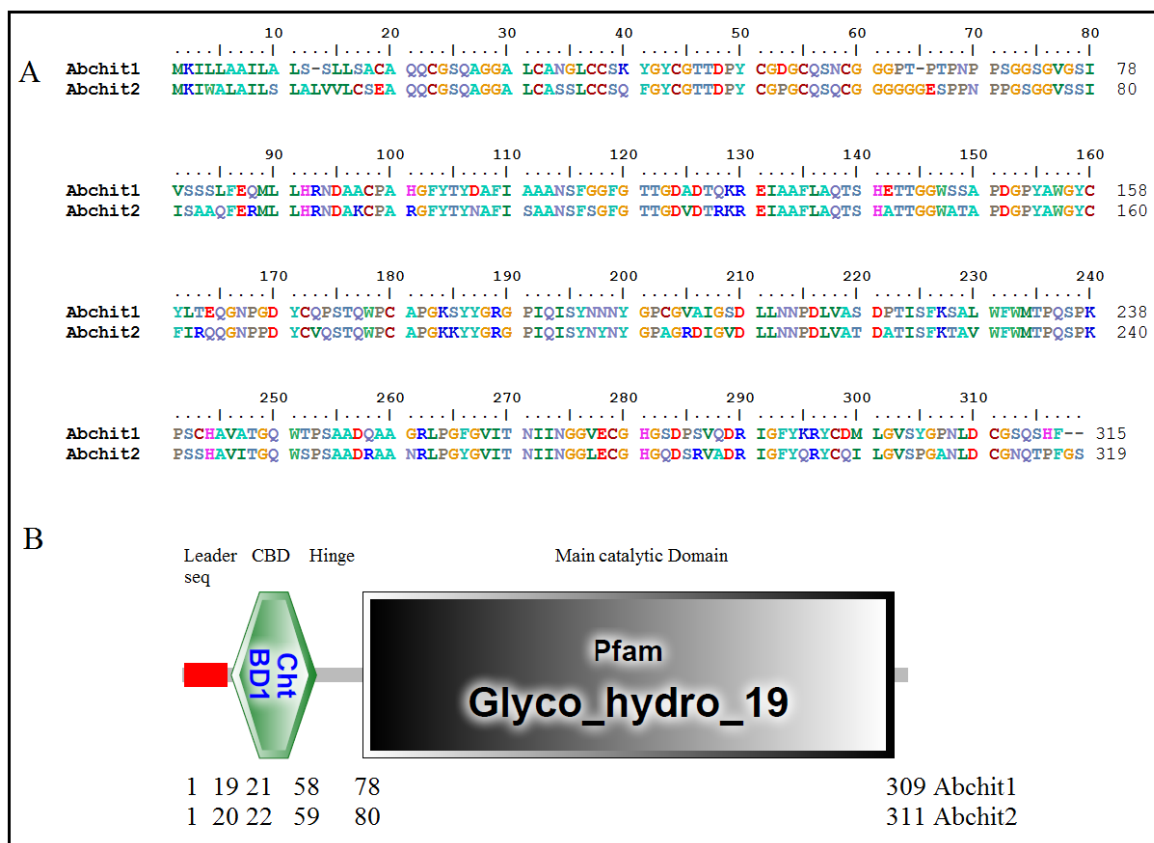
Fig.3 Positions of di-sulphide bond among various Cysteine residues of CBDs in Abchit1 and Abchit2 as determined by Prosite analysis

Fig.4 Expression profile of Abchit1 and 2 in different genotypes. In each data set 1, 2 and 3 represent different genotypes of *Aloe barbadensis*
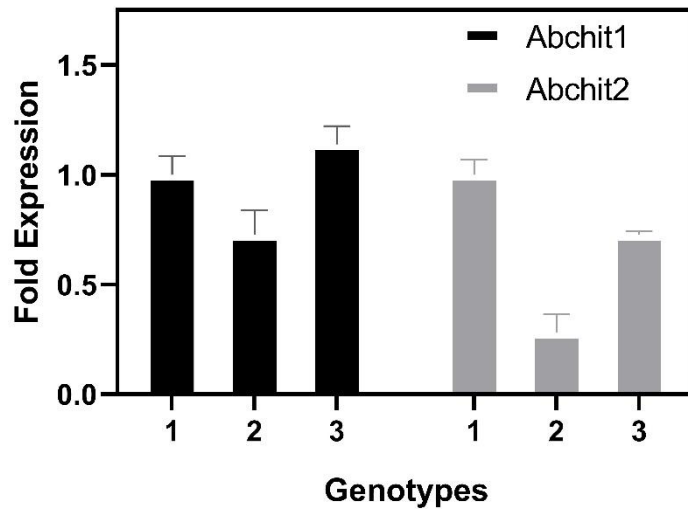
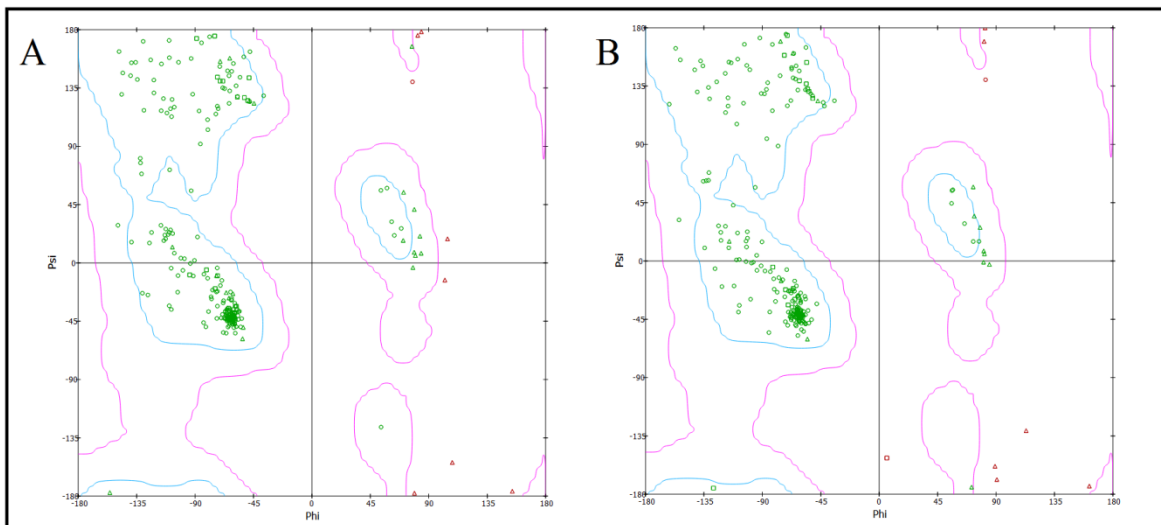**Fig.5** Ramachandran plot analysis of Abchit1 (A) and Abchit2 (B). Plot was generated using 3D models of both chitinases and Discovery studio prog (http://accelrys.com/products/collaborative-science/biovia-discovery-studio/)

Fig.6 Three dimensional structure of Abchit1 and Abchit2 along with substrate analyzed by PyMol (https://pymol.org/2/).A represents superimposition of both proteins. Abchit1 and Abchit2 are represented with red and green color respectively. B represents surface structure of Abchit1 and active site region between to lobes of protein
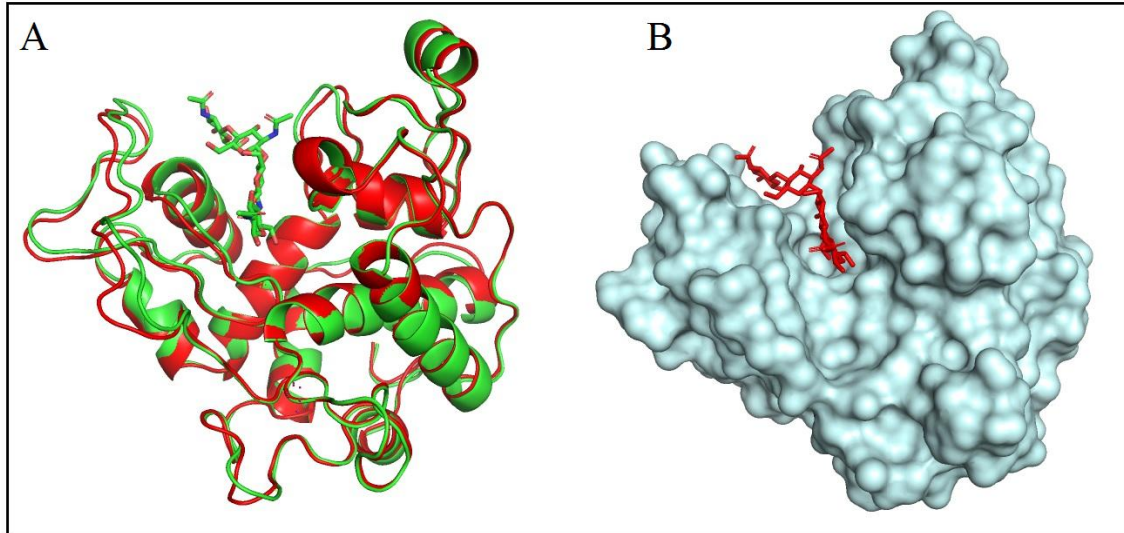
Fig.7 Abchit1 and Abchit2 amino acid residues interacting with oligo NAG substrate. Abchit1 and Abchit2 are represented by red and blue color respectively. Dashed line represents hydrogen bond formation
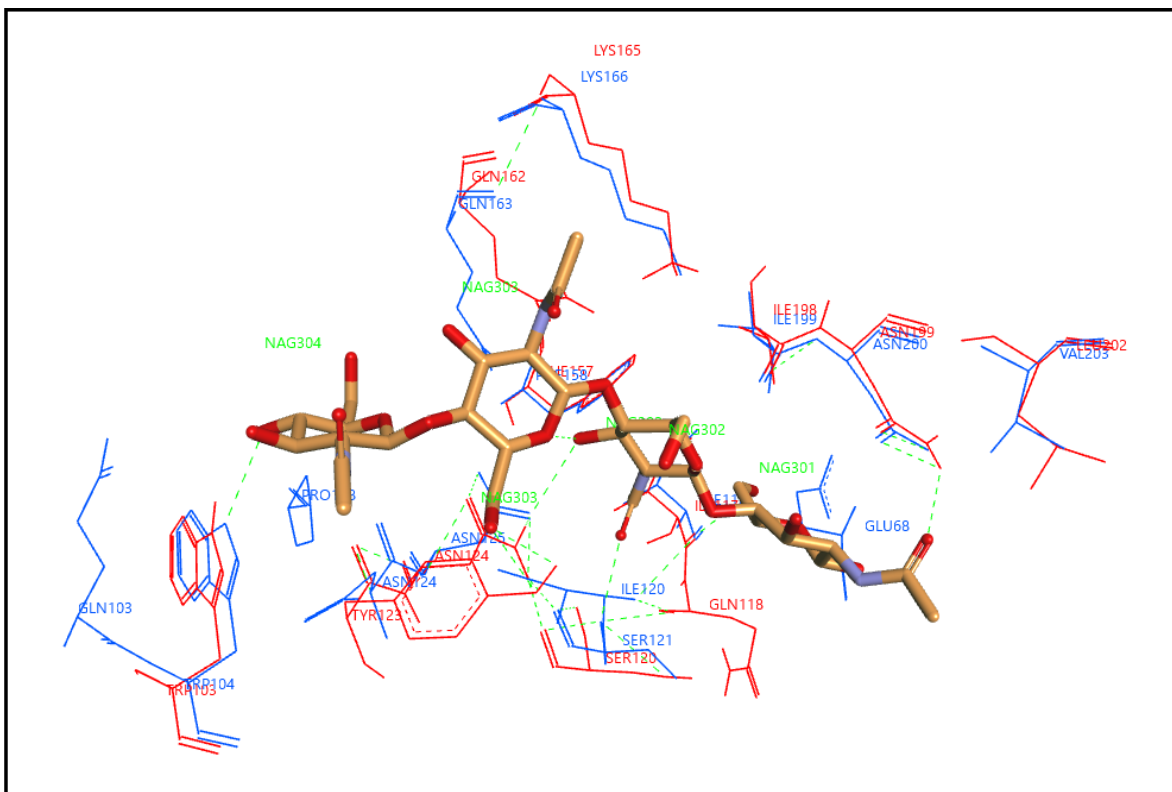
Fig.8 MD simulation: RMSD and radius of gyration: A represents RMSD time course of Abchit1 (black) and Abchit2 (red) during MD simulation. B represents change in Rg of Abchit1 (black) and Abchit2 (red) during MD simulation
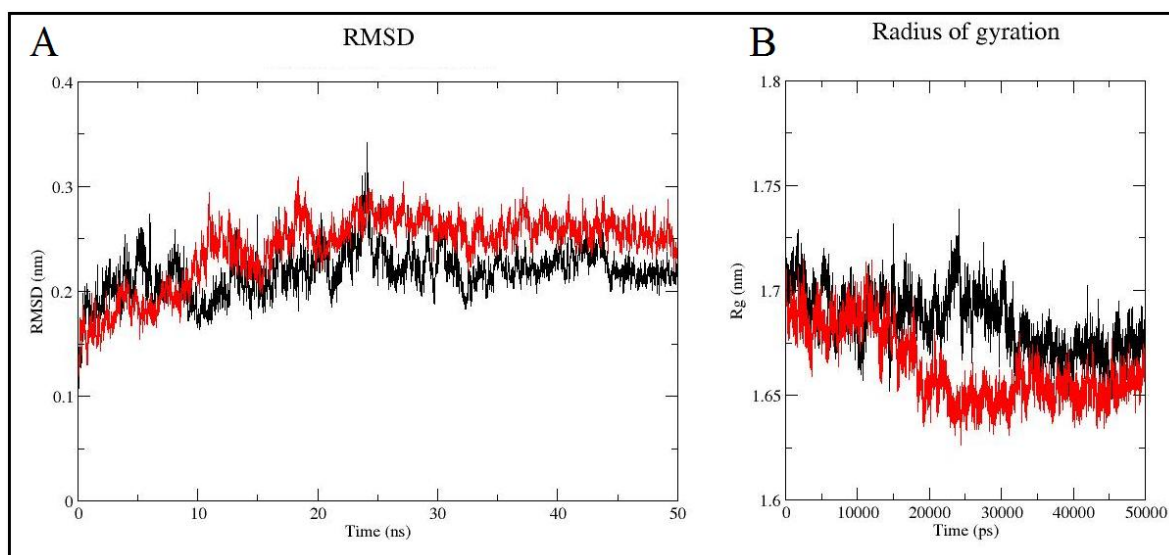

Fig.9 RMSF of Abchit1 (black) and Abchit2 (red) during 50 ns MD simulation. Analysis shows more RMSF in the loop regions of both proteins. Abchit2 shows more loop fluctuations as compare to Abchit1
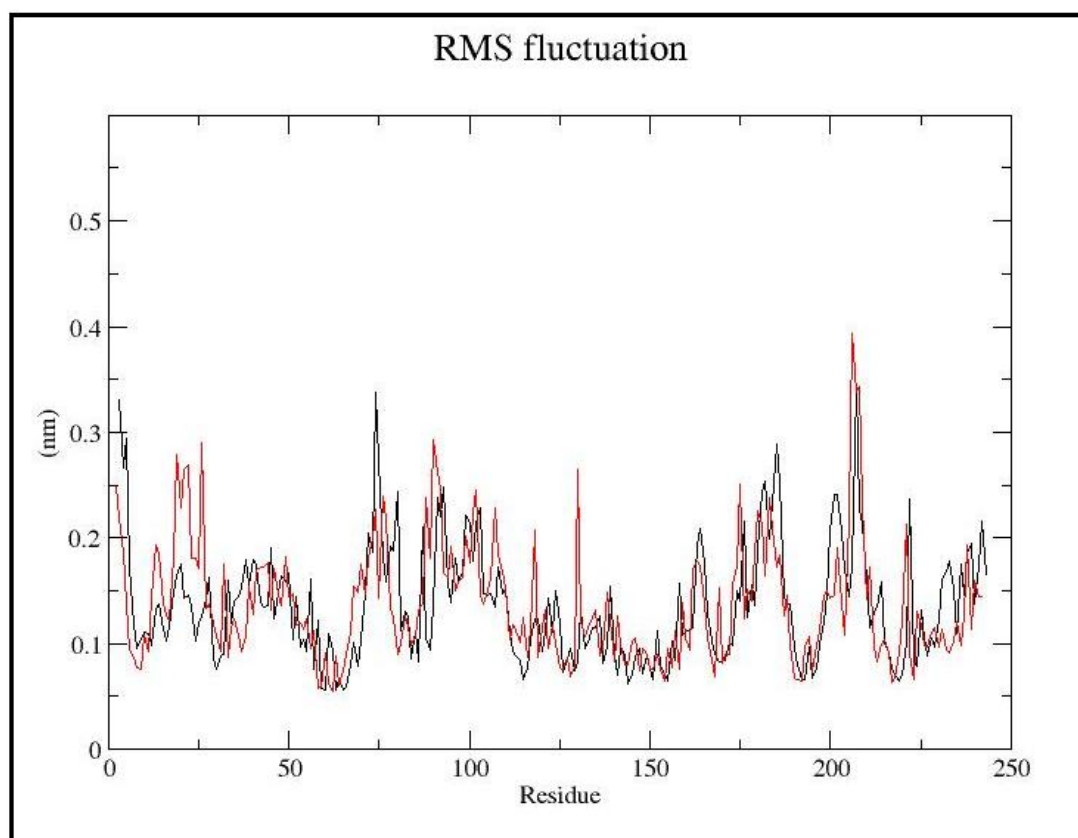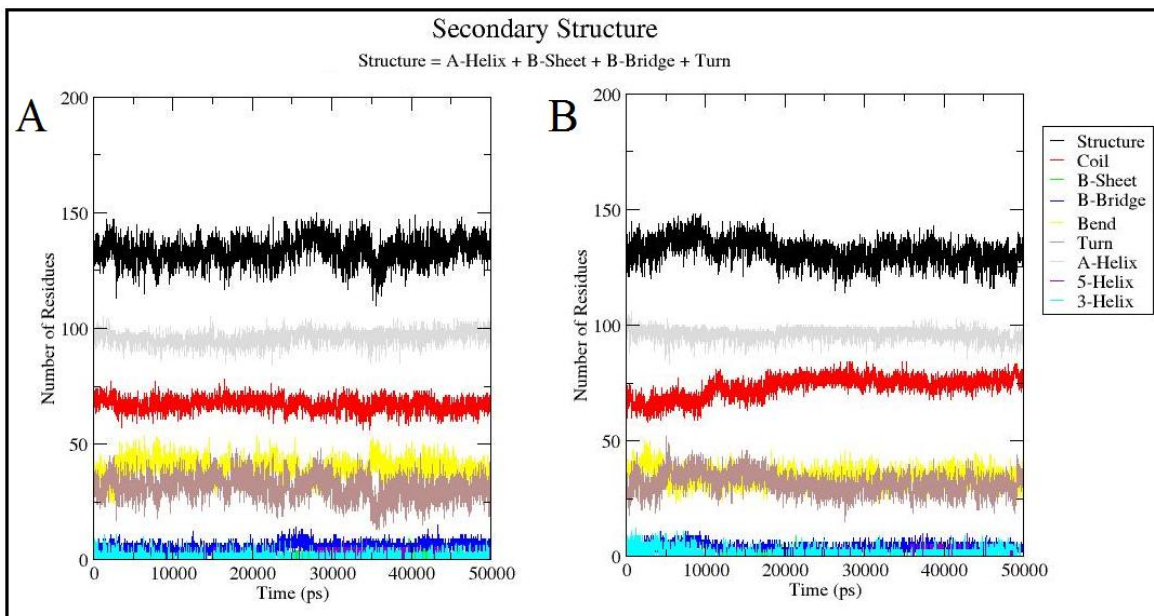
Fig.10 Secondary structure analysis of Abchit1 (A) and Abchit2 (B) during MD simulation. Figure represents total structure change and various structural classed of proteins

But, number of amino acids participating in structural bends formation showed some alterations. Results of present MD simulation indicated that both chitinases showed stable protein three dimensional structure, constant secondary structure but their loop regions shows some functions which depends upon differences in the primary structures of corresponding loops regions. Present study identify two class – I chitinases from *Aloe barbadensis*. These proteins show typical structural domain pattern consisting a leader sequence, a cysteine rich CBD domain and a main catalytic domain separated from CBD by a hinge region. The three-dimensional structure of these chitinases shows bi-lobed structure and the substrate binding site is present between these two lobes. Residues forming contacts with substrate were identified in both the chitinases which shows similar pattern of binding with NAG oligomer. MD simulation showed that the three dimensional structure of Abchit1 and Abchit2 is stable but the loop flexibility of Abchit2 is different from Abchit1. These differences in flexibilities are due to different primary structures of these proteins. Further biochemical analysis of these proteins may lead to development of new biocontrol agents or transgenic plants to cope with plant pathogens.

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