

Research Article

STRUCTURAL CHARACTERIZATION OF AN ENDO β -1,3-GLUCANASE OF FAMILY 81 GLYCOSIDE HYDROLASE (*CtLam81A*) FROM *CLOSTRIDIUM THERMOCELLUM*

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Abstract: The gene sequence of a family 81 glycoside hydrolase from *Clostridium thermocellum Cthe_0660*, (GenBank accession number: ABN51485.1) was retrieved from CAZy (Carbohydrate-Active Enzyme) database. The gene (2151 bp) encoding the catalytic module of *Cthe_0660*, an endo β -1,3-glucanase (*CtLam81A*) cloned, expressed earlier was used in this study. The secondary structure analysis of *CtLam81A* by PsiPred showed the presence of 23% α -helices and 23% β -strands whereas, CD analysis showed 24% of α -helices and 24% of β -strands. The secondary structure analysis by Circular Dichroism corroborated the PsiPred results. The 3D modeled structure of *CtLam81* generated by Swiss-Model server and energy minimized by Swiss-PDB viewer displayed an N-terminal α -sandwich domain, a $(\alpha/\alpha)_6$ domain and a short β -sandwich domain at C-terminal. The quality assessment of modelled structure by Ramachandran plot displayed 94% residues in favoured region, 4.6% in allowed region and 1.4% in outlier region. The superposition of *CtLam81A* modeled structure with its closest homologue *Bh0236* (PDB ID: 5t49) from *Bacillus halodurans* indicated that Glu515 acts as a catalytic base, while Asp438 acts as a catalytic acid. Multiple sequence alignment showed that these catalytic residues are conserved within the family 81 glycoside hydrolase. *CtLam81A* showed lowest binding energy against laminaripentaose and the amino acid residues, Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637 create the binding pocket at active site to hold the ligand.

Key words: Family 81 glycoside hydrolase; β -1,3-glucanase; Laminari-oligosaccharides; Circular Dichroism; Homology modeling; Docking; *Clostridium thermocellum*

Note: Coloured Figures are available on the Journal Website in "Archives" Section

Introduction

β -1,3-glucan is a major carbohydrate found in bacteria (*Alcaligenesfaecalis*, *Agrobacteriumrhizogenes*, *Agrobacterium radiobacter*), as a linear chain of β -1,3-linked glucose (McIntosh *et al.*, 2005) while in fungi (*Lentinusedode*, *Sclerotiumrolfsii*, *schizophyllum commune*) and algae (*Laminariadigitata*) a chain of

β -1,3-glucan branched either with β -1,6-linked glucose or with short chain of β -1,3-glucan (McIntosh *et al.*, 2005; Volman *et al.*, 2008). β -1,3-glucan is hydrolysed by β -1,3-glucanase enzyme, which are classified in to two types, exo- and endo-acting. Exo- β -1,3-glucanase (EC 3.2.1.58), acts on the terminal of chain and releases glucose ordimers (Mouyna *et al.*, 2013). These exo-acting enzymes fall into the glycoside hydrolase families 3, 5, 17 and 55. Endo- β -1,3-glucanase (EC 3.2.1.39) acts randomly on the β -1,3-glucans and releases mixture of oligosaccharides. Endo- β -1,3-glucanases falls in to families 16, 17, 55, 64, 81 and 128 of glycoside

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Received: February 13, 2018

Accepted: August 15, 2018

Published: August 20, 2018

hydrolase (GH). Thus, both GH17 and GH55 families contain β -1,3-glucan specific enzymes that have both, exo- or endo-activities. Family 81 glycoside hydrolases are known for their endo- β -1,3-glucanase activity and are widely distributed in plants, yeast, fungi, bacteria, archaea and viruses. Presently, family 81 GH contains 674 proteins distributed over bacteria, archaea, eukaryotes and viruses (<http://www.cazy.org/GH81.html>). All of the proteins in family 81 GHs share a common size of around 650 amino acids. Some of its members are biochemically characterized like β -glucan-binding protein (GBP) from *Glycine max* (Friegmann *et al.*, 2005), TfLam81 from *Thermobifida fusca* (McGrath & Wilson, 2006) and Eng2 from *Saccharomyces cerevisiae* (Martin-Cuadrado *et al.*, 2008). ^1H NMR spectroscopic analysis of GBP hydrolysed product revealed the retaining hydrolytic mechanism of this family (Friegmann *et al.*, 2005). So far only two enzymes, RmLam81A from *Rhizomucor miehei* (Zhou *et al.*, 2013) from *Bacillus halodurans* (Pluvnageet *et al.*, 2017) provide information about crystal structure solved from family GH81. Crystal Structure analysis of RmLam81A depicted the presence of β -sandwich domain at N-terminal and $(\alpha/\alpha)_6$ domain at C-terminal connected via additional domain between them. Similar type of domain was also found in BhGH81 protein. Interaction study of laminarin with BhGH81 revealed that the active site of this protein can accommodate maximum five glucose residues of β -1,3-glucan (Pluvnageet *et al.*, 2017). *Clostridium thermocellum* contains multienzyme complex, known as cellulosome. Cellulosome complex is made by a high affinity interaction between dockerin modules of the cellulosomal catalytic proteins and cohesin modules on the scaffoldins (Fontes and Gilbert, 2010). One such cellulosomal enzyme of *Clostridium thermocellum* is β -1,3-glucanase (*Cthe_0660*) belongs to family 81 GH. The catalytic component, CtLam81A of *Cthe_0660* was cloned and purified earlier (unpublished results) was used in the present study. In this study, the secondary structure elements of CtLam81A were determined by Circular Dichroism and also confirmed by PsiPred. The 3D structure of CtLam81A was modeled by homology modeling and ligand docking study was performed to identify the amino acid residues involved in the catalysis.

Material and Methods

Retrieval of amino acids sequence of CtLam81A - The gene sequence of family 81 glycoside hydrolase

protein of *Clostridium thermocellum* ATCC27405 having gene accession number ABN51895.1 and uniport ID A3DD6 was retrieved from CAZy database (<http://www.cazy.org/>). The boundaries of the family 81 GH catalytic domain (CtLam81A) in the amino acid sequence of *Cthe_0660* protein was identified by using the conserved domain database (<http://www.ncbi.nlm.nih.gov/cdd/>). The 2151 bp gene encoding CtLam81A protein was cloned, expressed and purified earlier in our laboratory (Unpublished results).

Secondary structure analysis of CtLam81A - Secondary structure of CtLam81A protein sequence for alpha helices, beta strands, turns and loops were predicted with the help of PsiPred v3.3 method (<http://bioinf.cs.ucl.ac.uk/psipred/>). The composition of secondary structure of CtLam81A was also determined by Circular dichroism (CD). The purified CtLam81A at a concentration 0.7 μM in 50 mM phosphate buffer, pH 7.5 was used for CD analysis. The CD spectrum was recorded on a spectro-polarimeter (Jasco J-815, Japan) at 25°C using 1 nm bandwidth over far UV region between 190 to 250 nm at scanning rate of 50 nm/min. The CD data were presented by difference in molar extinction coefficient ($\Delta\epsilon$, deciliter mol $^{-1}$ cm $^{-1}$) as a function of wavelength (Kelly *et al.*, 2005). Percentage of α -helix and β -sheet were measured by web based K2D3 software package (<http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/>) (Andrade *et al.*, 1993).

Homology modelling CtLam81A-The three dimensional structure of CtLam81A was modelled with the help of SWISS-MODEL server. SWISS-MODEL is a web based server, which automatically identify structural templates based on BLAST analysis and generate 3-D protein structure (<https://swissmodel.expasy.org/>). The best modelled structure was selected based on the alignment score with templates.

Model refinement and quality assessment - The best modeled structure was energy minimized by Swiss-PDB viewer. Swiss-PDB viewer comprises a version of GROMOS 43B1 force field (Van Gunsteren *et al.*, 1996). This force field allows the evaluation of the energy of structure as well as it repairs the distorted geometry through energy minimization. Then the quality of energy minimized structure was checked by plotting the Ramachandran plot using RAMPAGE server (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>). This modeled structure was also validated by using the Verify-3D program

(http://services.mbi.ucla.edu/Verify_3D/), which analyzes the compatibility of atomic model with the amino acid sequence (Liithy *et al.*, 1992).

Prediction of active site and mechanism of action - The amino acid residues in the active site are generally conserved in the proteins of the same family. To identify the amino acid residues at active sites, CtLam81A was structurally aligned with the structure of BhGH81 (PDB ID- 5T49) using Pymol. To confirm whether these residues are conserved within family 81 GHs, multiple sequence alignment (MSA) was performed with the already characterized homologous proteins of the family 81 GHs *viz.* *Bacillus halodurans* GH81 (BhGH81; Q9KG76), *Thermobifida fusca* Lam81A (TfLam81A; AAZ56163), *Glycine max* Gbp (GmGbp; BAA11407), *Aspergillus fumigatus* EngA (AfEngA; AAF13033), *Saccharomyces cerevisiae* Eng2 (ScEng2; AAB82378) and *Rhizomucor miehei* Lam81 (RmLam81A; KC847083). The percentage similarity between amino acid sequences of CtLam81A with its homologous proteins from family 81 GH was analysed by BLAST tool (Altschul *et al.*, 1990). MSA was performed with the help of CLUSTALW program (<http://www.genome.jp/tools-bin/clustalw>) and the conserved amino acid residues were viewed by ESPript 3.0 (<http://escript.ibcp.fr/EScript/EScript/>). To study the mechanism of hydrolysis the distance between the two catalytic amino acids (Asp438 and Glu515; from C_atoC_a) of CtLam81A was measured in the molecular visualization system PyMol 2.0.

Molecular dynamic simulation of CtLam81A modeled structure - Molecular dynamic (MD) simulation of CtLam81A was performed by using GROMACS v 5.14 (Berendsen *et al.*, 1995). GROMOS96 53a6 force field was used to calculate the Protein forces, where the protein CtLam81A was placed within in a cubic box of single point charge (SPC) with water molecules. The CtLam81A protein charges were neutralized by adding the 15 numbers of counter ions (Na⁺). Then the whole system was equilibrated for 500 ps in NVT ensemble (constant number of particles, volume and temperature) was carried out for restraining the solute atoms. This system was again equilibrated for 500 ps by NPT ensemble (constant number of particles, pressure and temperature) twice, once with restraints and then without restraints. Production run was performed for 30 ns with NPT ensemble adopting a 2 fs integration time. The linear constraint solver (LINCS) algorithm (Hess *et al.*, 1997) was employed

to constrain the bonds associated with hydrogen atoms and radius of gyration. Throughout the production run the modeled CtLam81A structure was analyzed as a time dependent function to ascertain its stability in the solvent system. The variation in the CtLam81A protein backbone (root mean square deviation, RMSD) was estimated by the least square fitting method.

Biding interaction analysis of CtLam81A with the ligands - Molecular docking of CtLam81A with the laminari oligosaccharides was performed by using SwisDock, the web based server (<http://www.swissdock.ch/docking>). Laminaribiose, laminaritriose and laminaritetraose were downloaded from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Laminaripentaose and laminarihexaose were generated using the GLYCAM server (Kirschner *et al.*, 2008). The modelled CtLam81A was saved in PDB format and the ligands were saved in Mol2 file format for docking analysis in the Swiss Dock tool. Swiss Dock generates large number of ligand binding results. The enzyme-ligand docked complex showing the strongest binding with maximum of negative binding energy were selected. This ligand bound structure was downloaded and visualized in PyMol 2.0. The depiction of ligand interaction with the amino acid residues of the protein was generated using the PDBsum Generate tool (<https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html>).

Results and Discussion

Molecular architecture of CtLam81A

CtLam81A from *Clostridium thermocellum* contains an N-terminal catalytic domain and a type I dockerin domain at the C-terminal connected via linker. The dockerin domain binds to cohesion domain, located on the scaffoldin protein in cellulosome complex (Ding *et al.*, 2001). The 2151 bp gene encoding family 81 glycoside hydrolase (CtLam81) and linker was cloned in pET-28a(+) vector, expressed in *E. coli* BL21 cells and purified (Unpublished results). Total length of purified protein (CtLam81A) is 740 amino acids consisting of 23 amino acids of pET28a(+) vector, 660 amino acids of catalytic domain and 64 amino acids of linker at the C-terminal. The theoretical molecular size of CtLam81A is 82.4 kDa.

Secondary structure analysis of CtLam81A

Secondary structure of CtLam81A predicted by PsiPred tool showed the presence of 14 α -helices

(23%), 31 β -strands (23%) and 45 random coils (54%) (Fig. 1). CtLam81A showed almost an equal amount of β -strands and α -helices. The N-terminal of CtLam81A showed the predominance of β -strands and the C-terminal showed the abundance of α -helices. The predicted secondary structure was also confirmed by circular dichroism analysis of purified CtLam81A. The CD spectrum of CtLam81A was analysed with the help of K2D3 server, which gives the percentage of α helix and β strand by comparing the available secondary structures of known proteins (Louis Jeune *et al.*, 2012). This analysis showed that CtLam81A contains 24% α -helices and 24% β -strands (Fig. 2). The results of CD analysis corroborated with the secondary structure predicted by PsiPred method. This distribution of secondary structure elements is common in family 81 GH (Zhou *et al.*, 2013).

Homology modeling and structure validation

The web based server SWISS-MODEL generated the 3-D model structure of CtLam81A by using the structural template of protein BhGH81 (PDB ID - 5T49) having 42% sequence identity with query coverage 100%. The overall structure of the modelled CtLam81A (Fig. 3A) consisted of three domains, N-terminal β -sandwich domain I (red colour), an $(\alpha/\alpha)_6$ barrel domain II (green colour) and a short β -sandwich domain III (yellow colour) at C-terminal. This modeled structure was energy minimized and validated before docking analysis. Ramachandran plot of CtLam81A modeled protein showed that 94% of the residues found in the favourable region and 4.6% of residues found in allowed region and only 1.4% residues (Phe177, Thr189, Lys221, Asn465, Asp481, Thr592, Ile661 and Pro663) was found in outlier region (Fig. 3B). This indicated that the amino acid residues in the modelled CtLam81A occupied favourable phi (ϕ) and psi (ψ) backbone dihedral angles. Verify_3D result showed that 95.25% of the residues in modeled structure had an averaged 3D-1D score ≥ 0.2 , which indicated the compatibility of amino acids with the modelled structure (Fig. 3C). ProSA result of modelled CtLam81A indicated that the protein is error free and reside in the x-ray zone with Z-scores of -9.88 (Fig. 3D).

Catalytic cleft and mechanism of catalysis of CtLam81A

The amino acids residues involved in the catalysis and in substrate binding were explored by

superimposing the CtLam81A modelled structure with crystal structure of BhGH81 (PDB ID - 5T49) having RMSD, 0.495 Å as shown in Fig. 4A. The catalytic core of CtLam81A existed within $(\alpha/\alpha)_6$ barrel domain of the protein. The superposition of modelled structure of CtLam81A (shown in green colour) with BhGH81 (shown in cyan colour) showed that β -sheet and α -helix of CtLam81A aligned well with the β -sheet and α -helix of BhGH81 (Fig. 4A). However, some loop regions (43–65 amino acids, 96–105 amino acids, 214–222 amino acids and 296–302 amino acids) of the CtLam81A modelled structure do not align with the BhGH81 structure. The analysis of superimposition showed that Glu515 acts as catalytic base and Asp438 acts as acid catalyst while Trp588 and Trp589 helps in binding the ligand (Fig. 4B). CtLam81A shared amino acid sequence similarity with homologous proteins from *Bacillus halodurans* GH81 (BhGH81; Q9KG76), *Thermobifida fusca* Lam81A (TfLam81A; AAZ56163), *Glycine max* Gbp (GmGbp; BAA11407), *Aspergillus fumigatus* EngA (AfEngA; AAF13033), *Saccharomyces cerevisiae* Eng2 (ScEng2; AAB82378) and *Rhizomucor miehei* Lam81 (RmLam81A; KC847083) as shown in Table 1. The multiple sequence alignment of CtLam81A showed that the catalytic amino acids are conserved in all family 81 glycoside hydrolases. Trp588 is conserved in both bacterial and plant proteins, while Trp589 is conserved in bacteria only (Fig. 5). Glycoside hydrolases catalyse the glycosidic bond, either by inverting or retaining hydrolytic mechanism. It has been found that, in the inverting mechanism, the average distance between two carboxyl groups of acid/base catalytic residues is 10.5 Å and in the retaining mechanism the average distance is only 5.5 Å (Zechel & Withers, 1999). The superimposition of CtLam81A structure with crystal structure of BhGH81 indicated that Glu515 is catalytic base and Asp438 is catalytic acid. The distance between these two catalytic residues was found to be 8.3 Å (Fig. 6), indicating the inverting hydrolytic mechanism for CtLam81A. The same inverting hydrolytic mechanism was also reported for β -glucan-binding protein (GBP) a member of family 81 glycoside hydrolases from soybean (Friegman *et al.*, 2005).

Molecular dynamics simulation of CtLam81A modeled structure

Molecular dynamic simulation of modeled CtLam81A was performed in order to analyse the structure stability and compactness of the structure

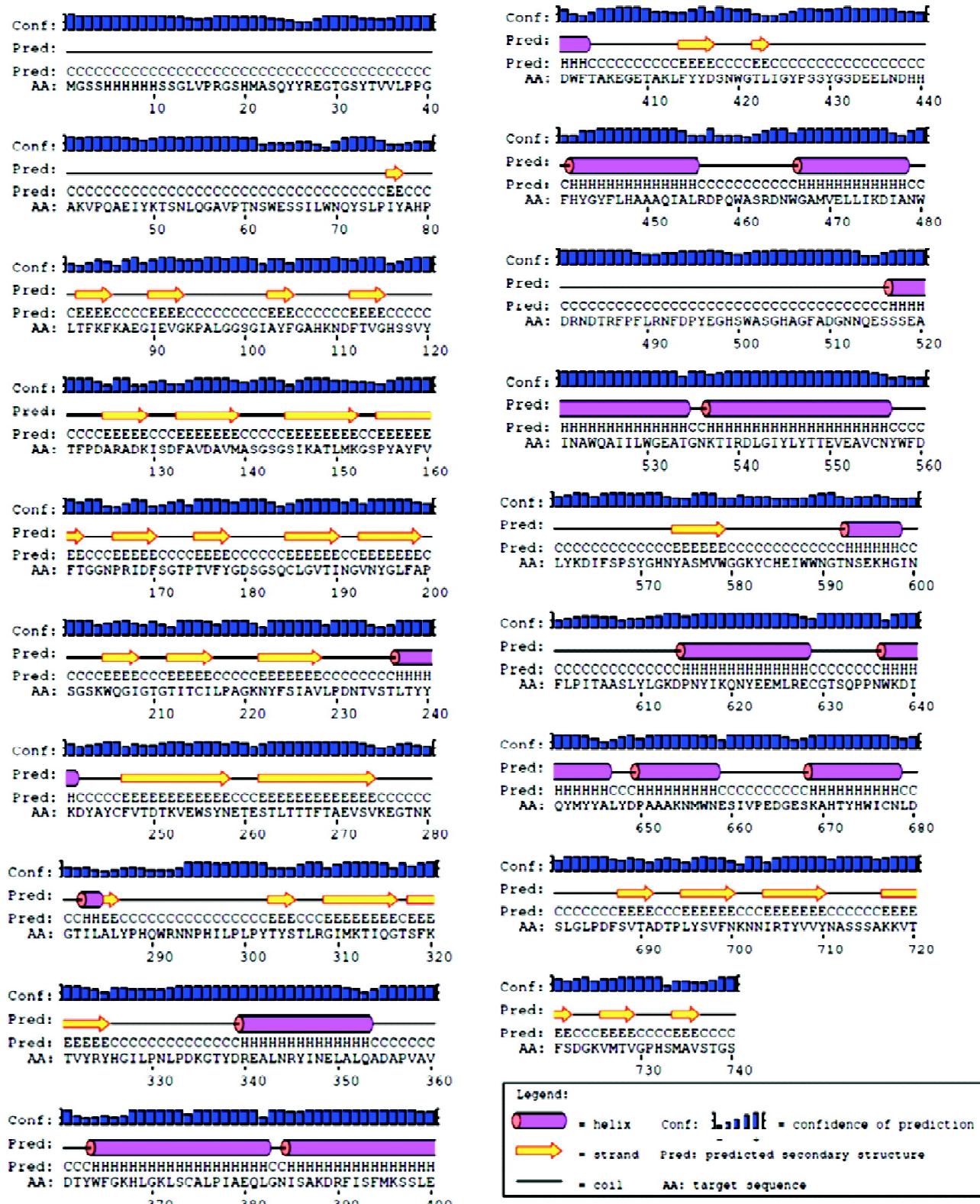


Figure 1: Secondary structure determination of *CtLam81A* by PsiPred server showing the amino acid residues involved in forming α -helix (cylinders), β -sheet (arrow) and random coil (continuous line).

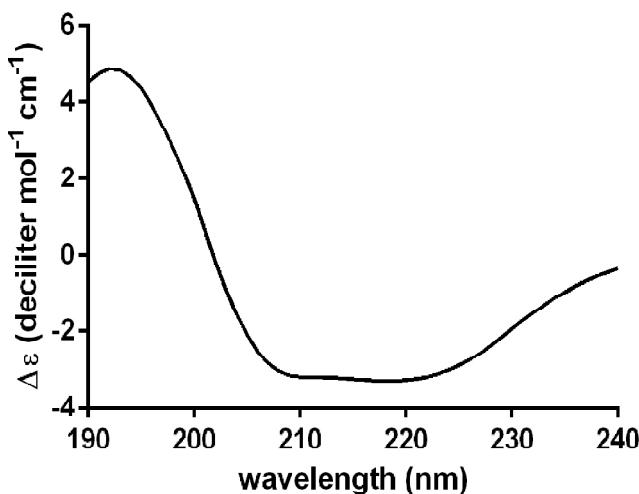


Figure 2: Circular dichroism (CD) spectrum of CtLam81A for determining the percentage of secondary structure elements. The CD data were presented by difference in molar extinction coefficients ($\Delta\epsilon$, decilitre $\text{mol}^{-1} \text{cm}^{-1}$) as a function of wavelength. The purified CtLam81A at a concentration 0.7 μM in 50 mM phosphate buffer, pH 7.5 was used for CD analysis. The CD spectrum was recorded at 25°C using 1 nm bandwidth over far UV region between 190 to 250 nm at scanning rate of 50 nm/min

over a 30 ns duration. The MD simulation results showed the fluctuation in RMSD value of CtLam81A up to 5 ns and thereafter the structure was completely stable till 30 ns. The overall deflection was 0.32 nm RMSD (Fig. 7A). The radius of gyration (R_g) of CtLam81A structure remained between 2.31 and 2.39 nm till the end of the run (Fig. 7B). Based on the MD simulation result of CtLam81A, it was concluded that the modelled structure of CtLam81A structure has a stable conformation.

Docking study of CtLam81A with ligands

The docking study of CtLam81A was performed in order to analyse the interaction of ligands with its active site. The binding energies of laminari-oligosaccharides with CtLam81A are shown in Table 2. Laminaripentaose showed the strongest binding with maximum of negative binding energy of -11.47 kcal/mol. The docking of laminaripentaose on surface of CtLam81A is shown in Fig. 8A. The non-reducing end of laminaripentaose is expressed as $-n$ subsite and the reducing end as $+n$ subsite. This result revealed that the catalytic cavity of CtLam81A can occupy maximum of five glucose

Table 1
Blast analysis of CtLam81A with its homologues from family 81

Organism	PDB ID	Query coverage (%)	Identity (%)	e-Value	Total Score
Bacillus halodurans C-125	5T4A	100	42	0	553
Thermobifidafusca YX-ER1	-	96	43	0	570
Glycine max	-	83	24	4e-35	130
Aspergillus fumigatus Af293	-	71	26	5e-35	130
Saccharomyces cerevisiae YPA84	-	64	27	2e-30	116
Rhizomucormiehei	4K3A	44	27	2e-25	112

Table 2
Binding analysis of CtLam81A with Laminari-oligosaccharides

Ligand	Binding free energy, ΔG (kcal/mol)	Residue making polar interactions	Residue making Hydrophobic interactions
Laminaribiose	-7.29	Glu515, Asp438	Tyr430, Gly431, Glu435, Asn437, Phe 441, Trp588
Laminaritriose	-8.24	Asn513, Asn437	Phe508, Glu435, Gly431, Ser503, Tyr430, Asp438, Trp558, Trp589, Asp438, Trp588, His442, Phe441, Glu515, Glu519, His442, Ile599
Laminaritetraose	-7.01	Tyr430, Trp588, Trp637	Trp589, Tyr353, Asp665, Trp364
Laminarpentaose	-11.47	Tyr363, Lys367, Tyr430, Asp438, Asp513, Glu519	Trp637, Asp665, Ile599, Leu602, Trp364, Glu515, Trp588, Gly431, Ala502, Phe441
Laminarihexasose	-10.18	Tyr363, Glu519, Asp438,	Trp588, Phe508, Asn437, Ala502, Glu515, Asn513, Tyr430 Phe441, Leu602, Lys367, Trp589, Asn590, Asn636, Trp637

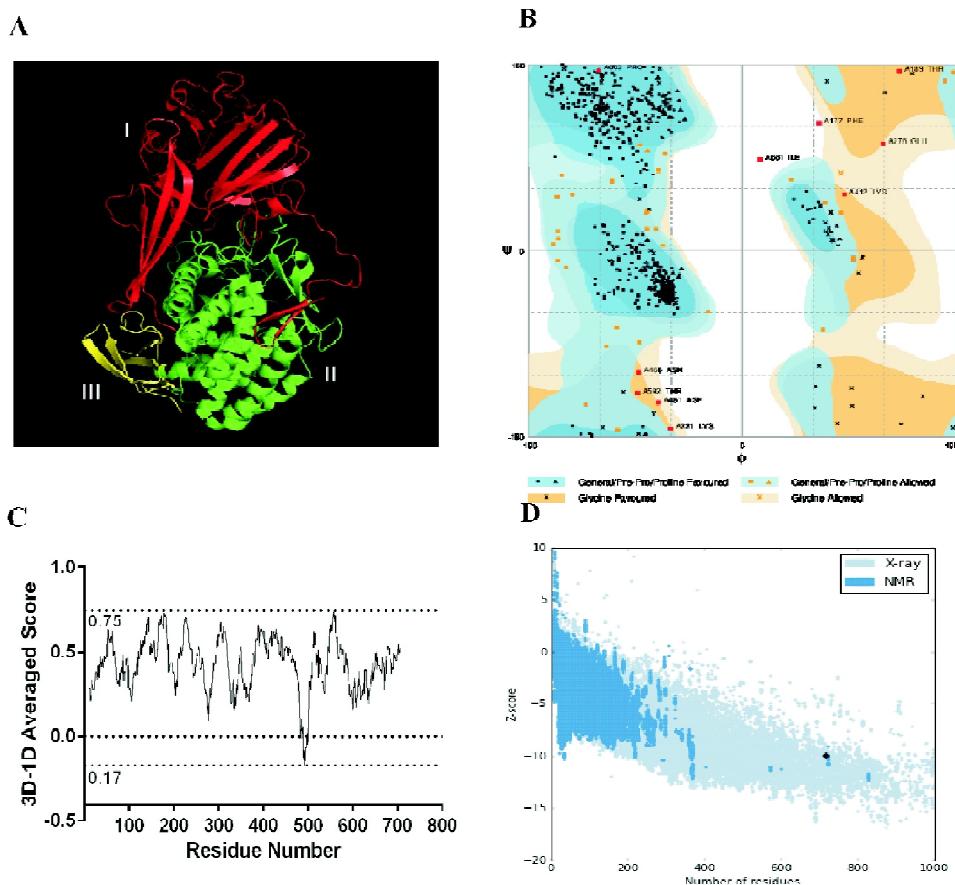


Figure 3: (A) Cartoon representation of modeled structure of CtLam81A displaying N-terminal β -sandwich domain I (red colour), an $(\alpha/\alpha)_6$ barrel domain II (green colour) and a short β -sandwich domain III (yellow colour) at C-terminal. Quality assessment of modelled CtLam81A by (B) Ramachandran plot of modelled CtLam81A, (C) Verify-3D and (D) Prosa web server

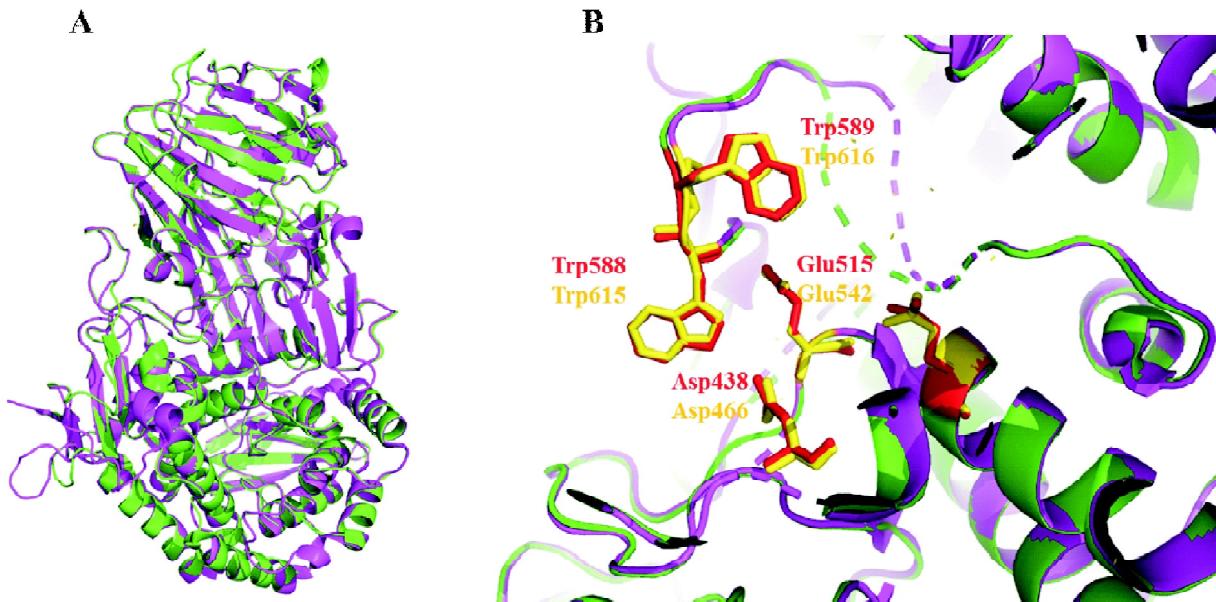


Figure 4: (A) Superimposition of *CtLam81A* modeled structure (green) with *BhGH81* crystal structure (cyan) and (B) Superimposition of active site residues of *CtLam81A* (red colour) with active site residues of *BhGH81* (yellow colour) by molecular visualization system PyMol 2

1 10 20

1 CtLam81A	MCSSHHHHHHSS...GLVPRGSHMASQYYREC.
2 BhGh81	XGSSHHHHHHSS...GLVPRGSHXASHAVSVG.
3 RmLam81MRFQV
4 TfLam81A	MS.....	HASRRRWRA....	TTSAATAALLCG...ALLTFPSAPAAAQVRLG.
5 AfEngA
6 ScEng2	MCYSRQAIPPPVPNRGGTNRGPPPLPPRANVQPPVCSSSENSSKPRENRVAGESLRTPS
7 GmGbp

30 40 50

1 CtLam81A	TGSYTVVILP	P.....	GAKVPO.....A.....EIY.....K
2 BhGh81	KGSYATEEPE	IDFGGI.....NDPGFRDQQGEPPATIYR
3 RmLam81	...IVAAATITMITSYI	GPVASQSTS	SDGDDLFVFVSVNFDPKSIFPEIKHPFEP...MYA
4 TfLam81A	...SGSITVILP	GASGPs.....D.....HTG.APVAPKV
5 AfEngAMGII.VSF	LKV	VVPLV....ANGQDVFVFPVS.....TGPNSQDDWLE....ERS
6 ScEng2	SSNPLADSQVN	SDNIFQSPV	L.SNLKAPPSSVFNKV.....QHPVPK...PNI
7 GmGbpMVNIQTNTSYIFPQTQSTVLPDPSKFFS

60 70 80 90

1 CtLam81A	TSNLQGAVPTNS	WESSION	WNQ.....YSLPIYAHPLTFKFKAEGLIE...VGKPA...LGG
2 BhGh81	SDRVTGPKXTNS	WWCGSLA	VDR.....FSXNQYPHPFSVRHRAEGLH...VFYDAPHNXVV
3 RmLam81	NTENGKIVPTNS	WISNIFYPSA	...DNLAPTTPDEYTLRLIDGYGGNN..PGLTIIRQPSAK
4 TfLam81A	TADFTQPVVTND	WWSSI	FQRYPGPNPYGENLYAHPLSFKAQAHGLE...VGYPDTPELVA
5 AfEngA	SVNTIDTPVEINK	FYCGLFLGTQTNNTIFTIHYPSVAVWKGGGTQSQSYGMAISHVESN
6 ScEng2	DDOSVDPLETNK	FYTNNMLDDNTOPIWTHPEYSIWFSRD...ELFGLAAANTLAS
7 GmGbp	SNLSSPLPTN	SFFQNFV	LKN....GDQQEYIHPEYLIKSSNSGL...SLSYPSRQASSA

100 110 120 130

1 CtLam81ASGI...AYFGAHKNDFT	VGHSS...VYTFPD	ARAADKISDFAVDAVM..
2 BhGh81	H...ENREA.GTW...HIHGAIGTDFT	IKHSG...TANFEQAVV	DDYNDWYVRGLLI..
3 RmLam81	VLGSYPPNDVPTDAGYMINSVVVDLR	LTSESEWSDVVPDR..	.QVTDWDHLSANLRLST
4 TfLam81A	..DGL...DGL...KQYTHSPDFV.	LGIHG...L.NAPA	AKVAGYSDWTVTADL..
5 AfEngA	IVAHGPVNTAIPGSPISYYVNPIGHISVI	LASE...	LGPSTVLTENPLPFSANAVLRP
6 ScEng2	Q.....RVFDTTTNPPRFYFNPTNIKSFVFKARE...	FVSSNDIKL	EFRDMKHMSMCLLM
7 GmGbp	VI.....FO.....VNPD...LT.	IASAPOGPKQGPPGKHLI	SSYSSDLSV.TL..

140 150 160 170 180 190

1 CtLam81A	.ASGSG5IKAT	LMKGSPYAY	FVFTGGNPRI	IDSFTGPTVYGDSG...SQCLGVIT.INGVN
2 BhGh81	.ENGAHQXAITYGVGSPYI	FVVEYEDGSAV	LDFDIAPDVWEVN...GHVIGFSTHDHKH	
3 RmLam81	PQDSNSYIDFP	IVRCMAYIAT	ANYNLTQPFQLSQHAIISVEADEKKSDDNTSTFSG.RKF	
4 TfLam81A	.SDGTRQLRITI	IQQCLEPFVY	ADVSGGPIVERFIAFFTWWKKS....GNAVGVIT.VNGH	
5 AfEngA	SASSSQSITIP	VVQCMGFVTGIYSN	LQPKIQSGVFFTAKMVTAGS....PR.TGIF	
6 ScEng2	SLSSSQFIEFP	LVQCMGFVIAIYHDLGFL	RSAVGFRSLERI.S....VN.ERYGK	
7 GmGbp	.DFPSGSNLSSFF	LVRGSPYI	TVSVTQP	TPL...SITIHSILSFS....SN.DSNTK

200 210 220 230

1 CtLam81A	YGDFAPS	GSKWQGIG	TGTI.....	TCILPAGKNYFSIAVLPDNTVSTLT
2 BhGh81	YAAFAPP	QONWSGIGSKTL	TNNADYIAIAKLP
3 RmLam81	ITMNDDPTSTI	IIYSLGDK	...PLELRKQDNSNLVASKR	TKWKTGIVIRVAKLPAP..EFET
4 TfLam81A	YALFAPS	GTITWSESD	TVF.....	TADVGGSGYASVALLPSP..DDFD
5 AfEngA	YSLSLLEDGTS	SWLLYATPDDGSDPQ...	OLASNSEIIIGPAGWGSTIQVA	KNPAGA.SGEK
6 ScEng2	YNIQLENNRNW	ILYLITSPDYSFPQDFQISLLD	SNTIISSSHKINGLICQLS...A.DSVP	
7 GmGbp	YTFQFNN	GQTWLLYATSPIKLNHTLSEITSNAFSGIIRIALLP	DSDSKHEA

240 250 260 270 280 290

1 CtLam81A	YYKDYAYC	FVTDIK	VEWS.YNETESTLTT	TFTAEVSVK.EGTNKG.TILALYPHQWRNNP
2 BhGh81	KFEQYAPP	SVRDAV	ADWT.YDEATGTVTT	TFEVTEAKVQGAPDG.TIFALYPHQYRH
3 RmLam81	LLDASRAV	WPTGGD	ISARSDDNGNGASYTI	TKWKTID...N...SNEAPLTYAYAHHTSID
4 TfLam81A	RYAPYAYS	FVTSTT	LTYD.YDPASATLTS	TYRVTTEAR.EGTAQG.TLLALYPHQWKETT
5 AfEngA	FYDNSSGV	YAVEGAVMGS	VSESTGTYSLMWAK...	AGKDAONTPLMFALPHHME
6 ScEng2	SIDMAAGCY	PVYCD	LSGQTVDDEHTFNYRF	NYTV...AGY.SQSGETLMLYALPHHKAAFT
7 GmGbp	VLDKYSSC	Y	PVSGKA	VFR...EPFCVEYNEWEK...K...DSGDL

300 310 320 330 340

1 CtLam81AHILPLPYTYST	TIRGIMKTI	IQGTSFKTVYRYH...	GILP...NLPDKGTYDREAL
2 BhGh81	SSSENQIL	LQNYQYEI	IRGTXIGLEGKRF	TTELTYP...GVLP...SLPDLGDYDRER
3 RmLam81	DSNVK.RTDM	TLOSATKGPMTALVG	NWTLRETELSPV.FWLPLQAAP...NPTTI
4 TfLam81ATALT.DLSYAS	SPRCPMRVV	EGDRFTTELTTH...	GILP...SLPTVDSADHQRL
5 AfEngA	ASTQSRATNI.TLRT	TTKGQATAV	I	GEYWTMVEPEPLISMCFAPWSVS
6 ScEng2	PEMQEREIAS.SLDS	TVKCLMTG	YLTNSFDM.QVQVQPEI	GFEPEVALSLNKKADY
7 GmGbp	NGDNDVKI	LEDLK	YKGSQD	UVGVGDSWVLKDPL...FV

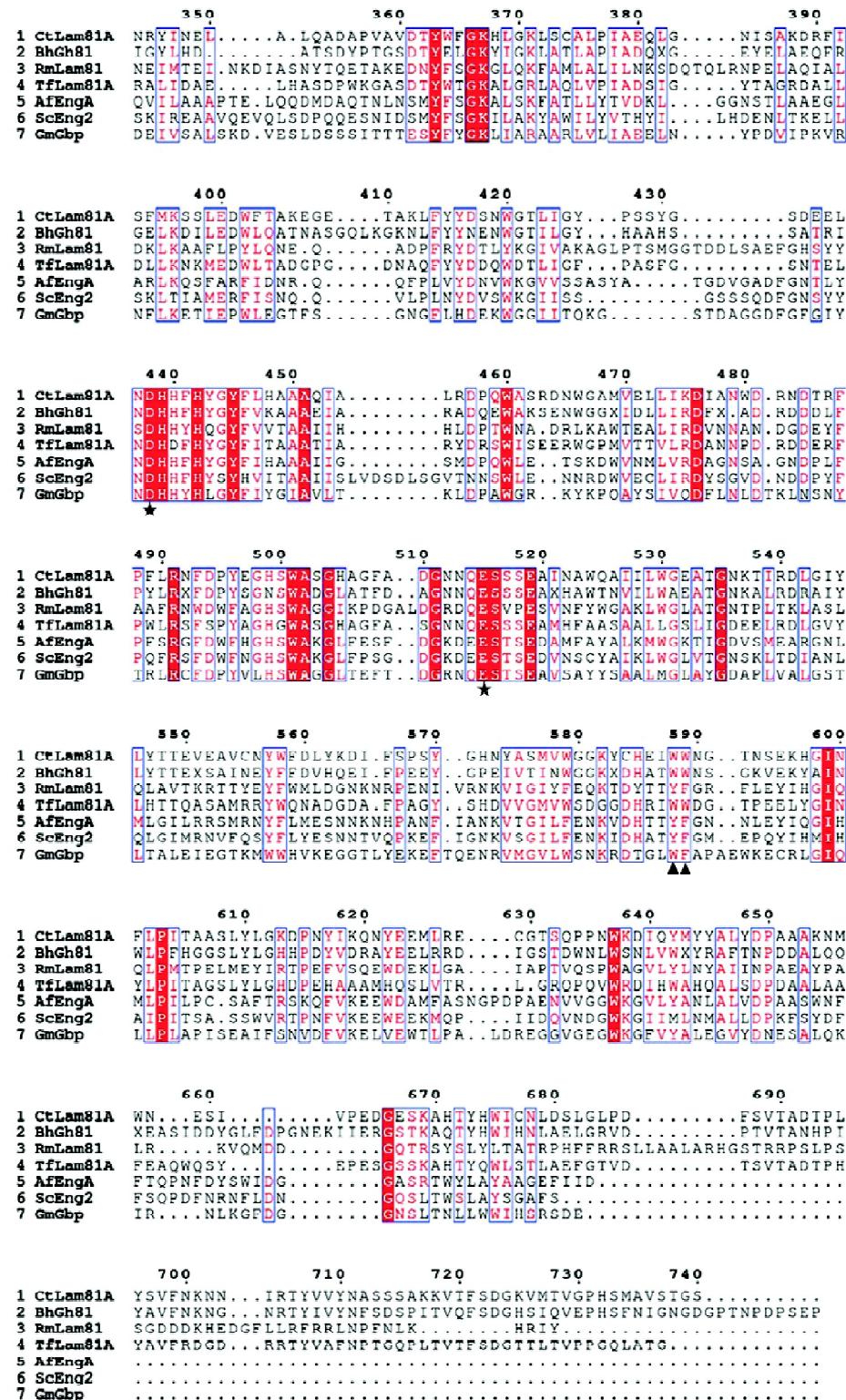


Figure 5: Multiple sequence alignment of CtLam81A with the homologous protein of the family 81 glycoside hydrolase viz. *Bacillus halodurans* GH81 (*BhGh81*; Q9KG76), *R. miehei* Lam81 (*RmLam81A*; KC847083), *T. fusca* Lam81A (*TfLam81A*; AAZ56163), *A. fumigatus* EngA (*AfEngA*; AAC13033), *S. cerevisiae* Eng2 (*ScEng2*; AAB82378) and *G. max* Gbp (*GmGbp*; BAA11407). The conserved amino acid residues are shown in red background and semi conserved residues are shown in box. This figure was generated by EsPrift3.0 (<http://esprift.ibcp.fr/>)

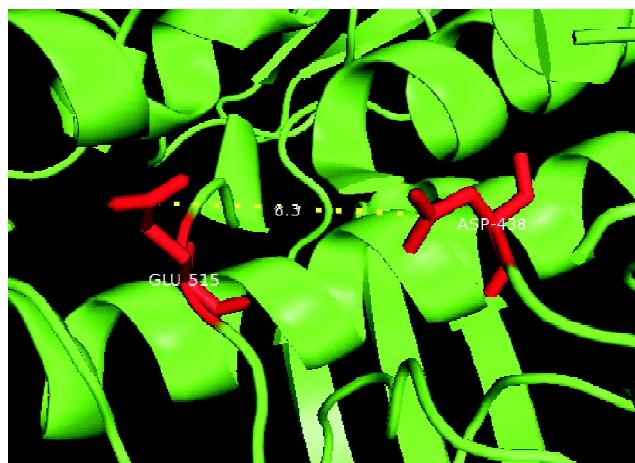


Figure 6: Determination of hydrolytic mechanism (Retaining or Inverting) of *CtLam81A* enzyme by measuring the distance (from C_g to C_g) between the acid catalyst (Asp438) and catalytic base (Glu515) of *CtLam81A* by molecular visualization system PyMol 2.

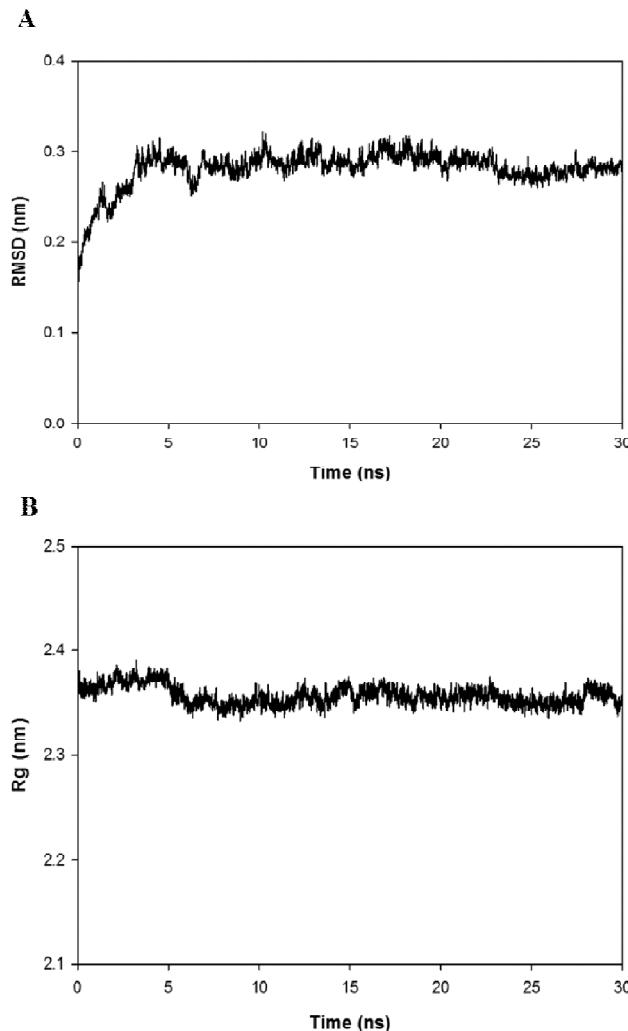


Figure 7: Molecule dynamic (MD) simulation of modeled *CtLam81A* showing (A) RMSD plot and (B) Radius of gyration plot

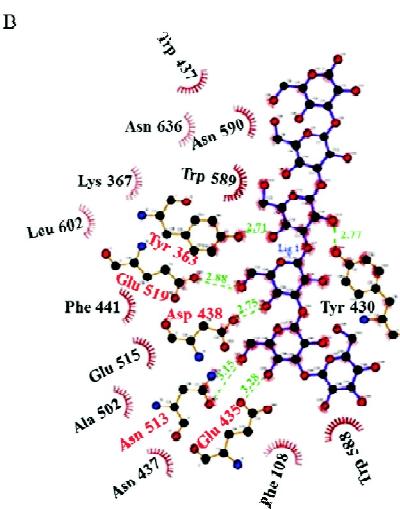
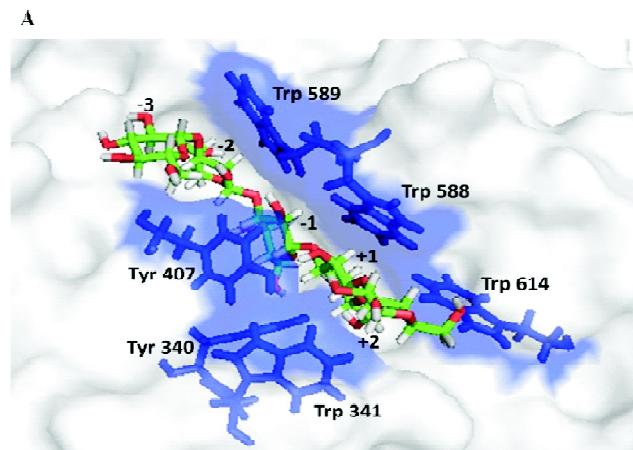


Figure 8: (A) Surface view of *CtLam81A* showing docking of Laminaripentaose with the amino acid residues of the catalytic cleft and (B) 2D Schematic presentation of Laminaripentaose interaction with the amino acid residues at active site of *CtLam81A*. Dashed lines show the hydrogen bonds and the amino acid residues shown in arc with spokes are making hydrophobic interactions with the ligand

residues of β -1,3 glucan. Similar results were reported for β -1,3-glucanase (*BhGH81*) from *Bacillus halodurans* (Pluvinageet al., 2017). The aromatic amino acid residues viz. Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637 in the catalytic pocket are involved in the holding of laminaripentaose. Trp 589 of *CtLam81A* lies near the -2 subsite of the laminaripentaose and Trp 588 lies near the +1 subsite, which helps in holding the ligand, as also reported for *BhGH81* from *Bacillus halodurans* (Pluvinageet al., 2017). The catalytic core residues, Tyr363, Lys367, Tyr430, Asp438, Asp513 and Glu519 of *CtLam81A* are making polar interactions with laminaripentaose (Fig. 8B), while, the residues Trp364, Gly431, Phe441, Ala502, Glu515, Trp588, Ile599, Leu602, Trp637 and Asp665 present at the

catalytic core are involved in hydrophobic interactions with the ligand.

Conclusion

The amino acid sequence of CtLam81A was analysed for secondary structure elements by PsiPred tool and CD. CD analysis showed 24% of α -helices and 24% of β -strands. Modelled structure of CtLam81A showed the presence of an N-terminal β -sandwich domain, a $(\alpha/\alpha)_6$ domain and a short β -sandwich domain at C-terminal. The active site lies in the $(\alpha/\alpha)_6$ domain of the protein. Superimposition of CtLam81A with BhGH81 and MSA confirmed that Glu515 is catalytic base and Asp438 is catalytic acid, which are 8.3 Å apart and involved in hydrolysing β -1,3-glucan by inverting mechanism. The docking study of CtLam81A with ligands showed the strongest binding energy against laminaripentaose, because the active site pocket of CtLam81A can occupy 5 glucose residues of β -1,3-glucan. The aromatic amino acid residues Tyr363, Trp364, Tyr430, Trp588, Trp589 and Trp637, Trp 589 and Trp 588 create a binding pocket for holding the ligand at the catalytic site.

Acknowledgements

This research work was carried out under bilateral joint DST (Ministry of Science and Technology, India) project (No. DST/INT/Portugal/P-14/2013) in collaboration with FCT Lisbon, Portugal. The authors thank Prof. T. Punniyamurthy, Head, Department of Chemistry, Indian Institute of Technology Guwahati for providing CD facility.

Abbreviations

GH, glycoside hydrolase; CD, Circular Dichroism

Conflict of Interest

The authors declare no conflict of interest.

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