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INSILICO ANALYSIS OF STRUCTURAL IMPACT OF N171A IN ENDO-B-N ACETYLGLUCOSAMINIDASES

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ABSTRACT

Endo-β-N-acetylglucosaminidases (ENGases) are important enzymes having dual specificity with an ability to catalyze hydrolysis and transglycosylation reactions. Because of their potential for synthesis of glycopeptides, these enzymes have become the focus of intense research. In this study, Energy minimization and subsequent Molecular dynamic simulations were performed for 10ns timescale to understand the stability and conformational differences among ENGase orthologs using Desmond. We compared ENGase orthologous proteins of bacteria, plant and Humans. Since, there is no crystal structure of Plant and Human available, we used homology modeled structures [OPR] and for bacteria Artherobacterial ENGase (PDB 3FHQ) were used. The results obtained from RMSF Graphs clearly indicates that, when compared to bacterial ENGase, the plant and Human ENGases showed high flexibility all along protein structures. Further, there was a clear deviation in flexibility at catalytic domains of bacteria where as in the other two eukaryotes, the deviation in the flexibility was in the gate keeper domain and transglycosylation sites where amino acid tryptophan and phenylalanine were replaced with Aspargine and tyrosine respectively. This change is found to be naturally evolved and should be attributing higher transglycosylation rates in human and plants.

KEYWORDS: ***Artherobacter protophormiae,*** **Endo-β-N-acetylglucosaminidases, homology modeling, conformational differences.**

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INTRODUCTION

Endo-β-N-acetylgucosaminidases (EC 3.2.1.96) (ENGases) are the enzymes that hydrolyze the glycosidic bond GlcNAc β-1,4GlcNAc present in N-linked sugar chains in glycoproteins and release the N-glycan moiety leaving, GlcNAc attached to protein. N-linked oligosaccharides play crucial roles in the quality control, folding, ER-associated degradation (ERAD) and sub cellular trafficking of glycoproteins [1]. These are key enzymes in the processing event of free oligosaccharides in the cytosol [2].The enzymes of this type are found in bacteria [3–5], fungi [6,7], plants [8,9] and animals [10,11]. Endo-β-N-acetylgucosaminidases are categorized into glycoside hydrolase families GH18 and GH85 based on the similarity in amino acid sequence .In case of the enzyme from *Flavobacterium meningosepticum* (endo-F) [13] the transfer of oligosaccharide to glycerol occurs. Some ENGases of GH85 family along with hydrolysis also possess transglycosylation activity, i.e., the ability to transfer the released oligosaccharide moiety to a suitable acceptor other than water. Endo-A [22] can transfer a high-mannose type oligosaccharide to monosaccharides such as N-acetylglucosamine (GlcNAc) and glucose to form a new oligosaccharide [2,15]. Transglycosylation activity towards complex oligosaccharides is exhibited in ENGase from Mucor hiemalis (Endo-M)[16]. Endo-A and Endo-M can transfer an intact oligosaccharide to the acceptor to form a new glycopeptide or glycoprotein in a single step, making it a highly convergent chemoenzymatic approach [17–19]. A number of homogeneous natural and unnatural N-glycopeptides and glycoproteins are synthesized by this chemoenzymatic approach [20, 21, 22]. Recently an endo-β-N-acetylglucosaminidase from *Artherobacter protophormiae*(endo-A) was reported which has transglycosylation activity, while the hydrolytic activity still predominated [24]. The enzyme was also used for synthesis of neoglycoprotein in the process of transglycosylation [25].It was found that the transglycosylation activity can be enhanced by suppressing the hydrolytic activity of endo-A. Endo-A is specifically for high-mannose or hybrid type N-glycans and has been applied for the synthesis of high-mannose type oligosaccharides and N-glycopeptides [26-28]. Recent findings prove that synthetic oligosaccharide oxazolines (the mimics of the oxazolinium ion intermediate of the enzymatic reaction) could be used for Endo-A catalyzed transglycosylation. This approach has significantly expanded the scope of chemoenzymatic methods for glycopeptides and glycoprotein synthesis [29]. It was found that the highly activated sugar oxazolines corresponding to the modified N-glycans could serve as substrates for the Endo-A catalyzed transglycosylation. The discovery of several mutants, ENGase based glycosynthase, including EndoA-N171A, could promote transglycosylation with sugar oxazolines of natural N-glycans. They lack the ability to hydrolyze the product, and thus enabled the synthesis of homogenous glycoproteins carrying full size natural N-glycans [30-32]. Based on the experimental evidence, to understand the structural changes linked with the dual catalytic activity of ENGase molecular dynamic simulation studies were taken. The protein structure of ENGases of orthologous proteins of Human, Arabidopsis and Artherobacter was compared to understand the structural differences and how it influences the activity of these enzymes. To explore the structural and conformational differences at deeper lever, molecular dynamics were done on these protein orthologous structures to know how the sequence difference effect on structure and how these structural difference effects on conformational changes further change or retain the activity of the ENGase orthologs.

Methodology

2.1 Dataset collection

As ENGases of Human and Arabidopsis does not have crystal structures, these structures were built through homology modeling approach with help of a prime module of schrodinger software [39]. The Artherobacter ENGase crystal structure 3FHQ is obtained from pdb and this structure was used as a template in homology modeling of other ENGases. This

modeled ENGases of Human and Arabidopsis proteins along with Artherobacter were docked with GlcNAc-Asn and finally these docking complexes were taken for simulation study.

2.2 Protein preparation

ENGase of *Artherobacter protophormiae, Human and Arabidopsis* protein preparation was done by using protein preparation wizard of Maestro software [33]. The proteins were treated by adding the missing hydrogens, by assigning proper bond order and by deleting all hetero atoms like catalytic ligand including water molecules. The H-bonds were optimized using sample orientations. All the polar hydrogens were observed. Finally, the Endo-A protein structure was minimized to the default Root Mean Square Deviation (RMSD) value of 0.30 employing OPLS_2005 Force field. The final low energy conformation protein was used for mutational analysis. Mutation at respective position and modeling was done using schrodinger software [35].

Molecular Dynamics

Desmond Molecular Dynamics system [34,35,37] with Optimized Potentials for Liquid Simulations (OPLS) all-atom force field 2005 [36,38] was used to perform MD simulations of modeled mutant and native form, which were prepared using protein preparation wizard of Maestro interface [33]. Prepared structures were then uploaded to Desmond set up wizard for MD simulations. The protein structures were prepared by the addition and optimization of hydrogens, disulphide bonds were generated, water molecules removed and the protein terminals were capped. Prepared protein molecules were solvated with TIP4P water model in a cubic periodic boundary box to generate required systems for MD simulations. Systems were neutralized using appropriate number of counter ions. The distance between box wall and protein complex was set to greater than 10Å to avoid direct interaction with its own periodic image. Energy of prepared systems for MD simulations was minimized up to maximum 5000 steps using the steepest descent method until a gradient threshold (25 kcal/mol/Å) is reached, followed by L-BFGS (Low-memory Broyden-Fletcher-Goldfarb-Shanno quasi-Newtonian minimizer) until a convergence threshold of 1 kcal/mol/Å was met. The systems were equilibrated with the default parameters provided in Desmond. Further MD simulations were carried on the equilibrated systems for desired period of time at constant temperature of 300 K and constant pressure of 1 atm with a time step of 2fs. Smooth particle mesh Ewald method was used to calculate long range electrostatic interactions in MD simulations. 9Å cut-off radius was used for columbic short range interaction cutoff method. The modeled ENGase protein was prepared for MD simulations using the parameters discussed above. The system was then continuously simulated at 10ns. Stability of docking of the modeled mutant and native proteins was also investigated using MD simulations. The modeled mutant and Native protein structures were simulated for 10ns time period using similar parameters as described above.The root mean square deviation (RMSD) for both the modeled mutant and protein structures was calculated for the entire simulation trajectory with reference to their respective first frames. RMSF analysis were carried out for all the frames of 10ns MD simulation of ENGase native orthologous and mutant structures.

RESULTS

Molecular dynamics simulations of Artherobacter , Human and Arabidopsis ENGase protein

To analyze the stability of Artherobacter (3FHQ), Human and Arabidopsis ENGase protein, RMSD of its backbone and ligand was plotted as the time dependant function of MD simulations [Figure 1]. Fluctuation in backbone of Artherobacter ENGase [Figure 2] during the simulations was recorded up to 1.8 Å. After 8ns of MD simulations, backbone was found to fluctuate around 0.2Å which persisted till the end of 10ns simulation. The standard deviation (SD) in RMSD for whole simulation process was found as 0.6 which was comparatively higher than 0.3 for the last 10 ns of simulation time. The RMSD plot of Human ENGase [Figure 3] protein showed fluctuation in backbone of modeled protein during the simulations was

recorded up to 4.5 Å. After 8ns of MD simulations, backbone was found to not fluctuate around 0.5Å which persisted till the end of 10ns simulation. The standard deviation (SD) in RMSD for whole simulation process was found as 0.6 which was comparatively higher than 3Å for the last 10 ns of simulation time. Finally the RMSD plot of Arabidopsis ENGase the fluctuation in backbone of modeled protein during the simulations was recorded up to 1.8 Å. After 8ns of MD simulations, backbone was found to not fluctuate around 0.2Å which persisted till the end of 10ns simulation. The standard deviation (SD) in RMSD for whole simulation process was found as 0.6 which was comparatively higher than 0.3 for the last 10 ns of simulation time By comparing all three RMSD plots, it is seen that protein had more flexible backbone in the beginning of the MD simulations but as the simulations continued, protein tend to acquire a higher stable configuration. Ligand RMSD with respect to protein and its binding pocket showed highest fluctuation after 5-6ns the protein structures were stabilized. A low RMSD throughout the MD simulation and consistent RMSD at the end of MD simulation indicated that the predicted tertiary structure of Human and Arabidopsis homology modeled structures as well as the Artherobacter (3FHQ) structures have acquired a stable folding conformation. Measure of the Root mean square fluctuations (RMSF) is used to characterize the local changes and trace out the flexible parts in the protein structure. Hence, peaks in RMSF plots were highly flexible region in the protein structure along the simulation. From the RMSF, RMSD and intra H-bonds profiling data collectively, it can be said that among the modeled structures, Arabidopsis protein was one of the most stable confirmation and there was no deviation found after 5.6ns MD simulation.

Figure 1

Molecular dynamics simulation of Atherobactor ENGase at 10000 ps. (A) Time evolution of backbone RMSDs is shown as a function of time. (B) RMSF of the backbone carbon alpha over the entire simulation. (C) Time evolution of the secondary structural elements.

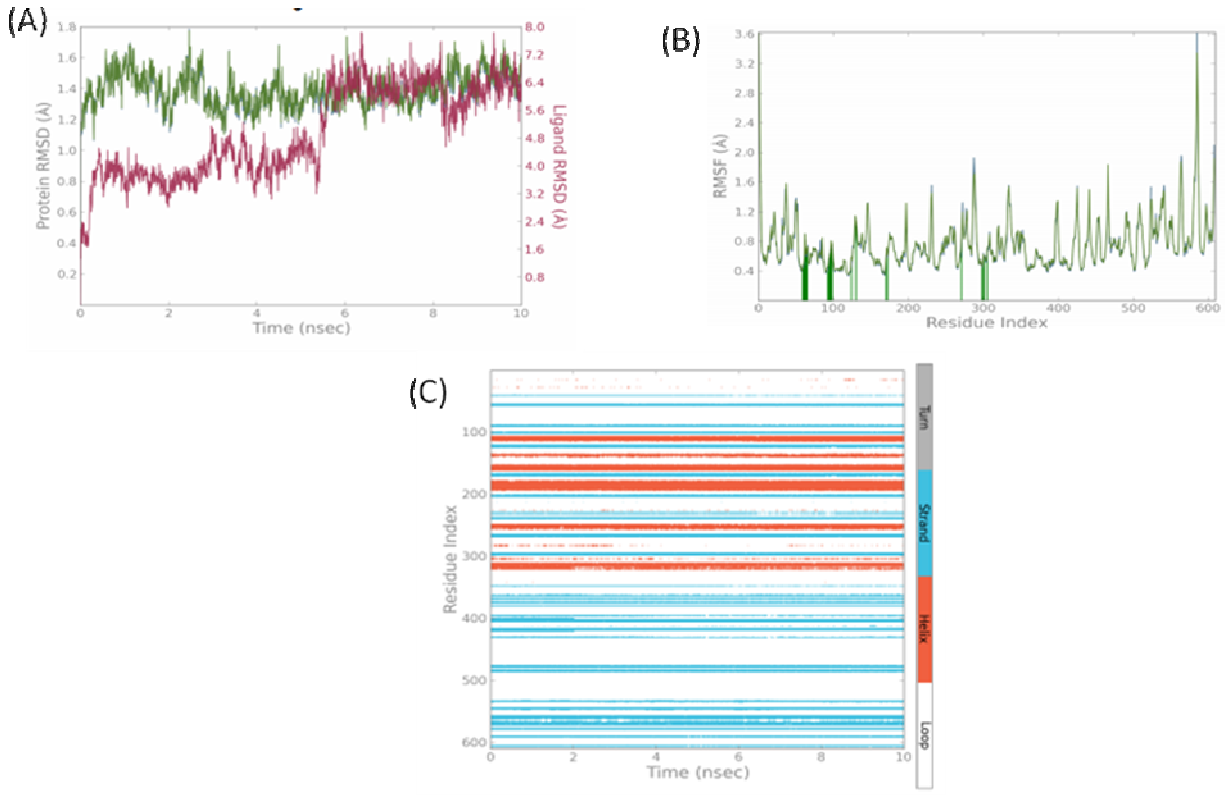

Figure 2

Molecular dynamics simulation of Human ENGase at 10000 ps. (A) Time evolution of backbone RMSDs is shown as a function of time. (B) RMSF of the backbone carbon alpha over the entire simulation. (C) Time evolution of the secondary structural elements.

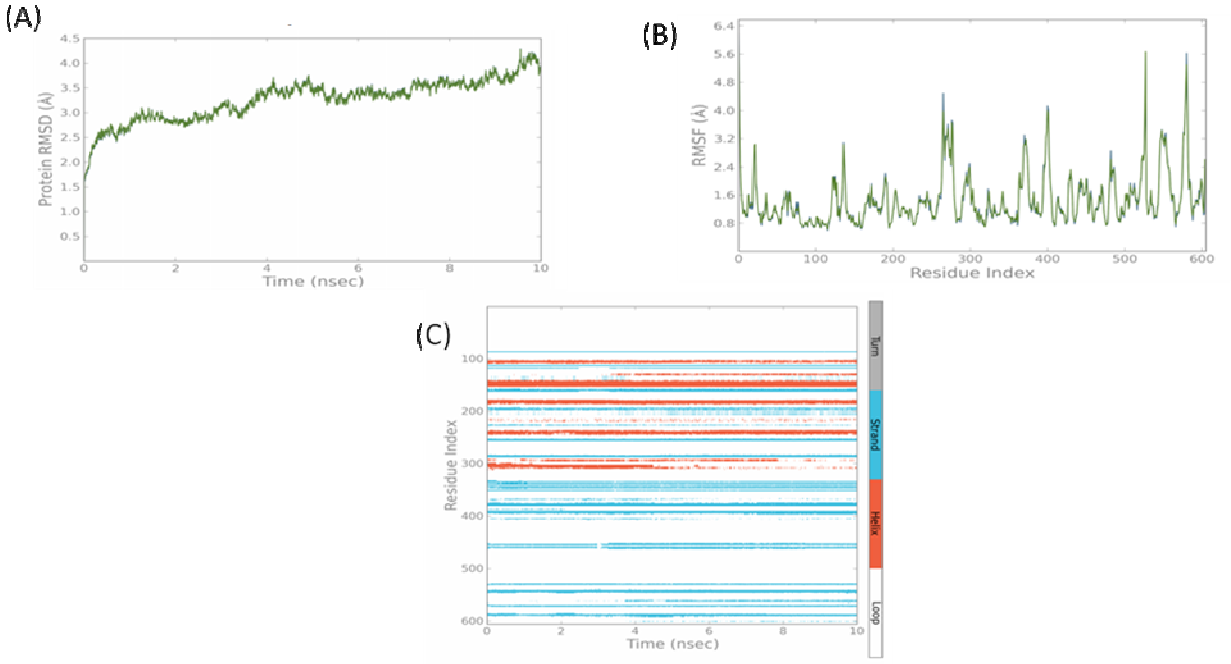

Figure 3

Molecular dynamics simulation of Arabidopsis ENGase at 10000 ps. (A) Time evolution of backbone RMSDs is shown as a function of time. (B) RMSF of the backbone carbon alpha over the entire simulation. (C) Time evolution of the secondary structural element.

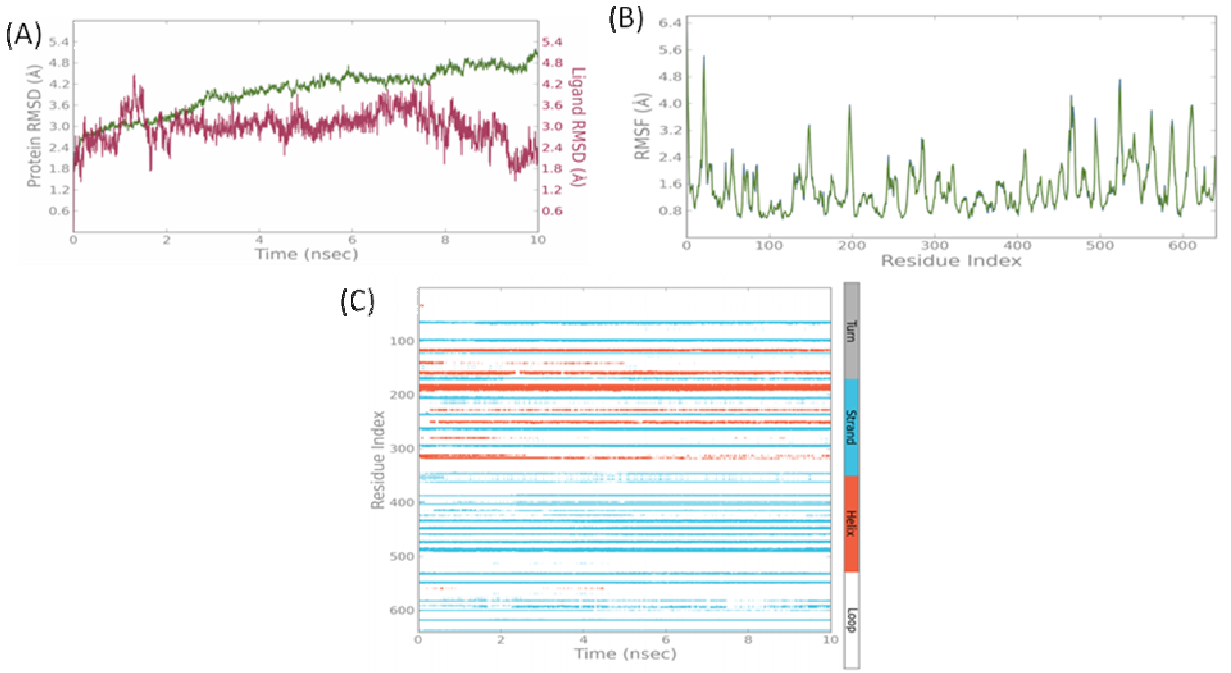

Table 1

The amino acid residue position and their flexibility deviations in three domains of Artherobactor, Arabidopsis and Human ENGase protein.

S.No	ENGase orthologs	Flexibility regions Domain 1 (~1 to ~350)	Flexibility regions Domain 2 (~351 to ~362 & ~387 to ~524)	Flexibility regions Domain 3 (~363 to ~386 & ~525 to ~611)
1	Atherobactor	~290 (2.1 Å)	Not Observed	C-terminal ~585 (3.6 Å)
2	Arabidopsis	N-terminal, ~10 (5.6 Å) , ~170 (3.9 Å) ~200 (4.1 Å),	~470 (4.8 Å), ~500 (4.8 Å)	C-terminal ~570,~590, ~610 fluctuated around 4.8 Å
3	Human	~270 (4.9 Å), ~280 (4.5 Å)	~400 (4.8 Å)	C-terminal-~520 (5.7 Å), ~550 (3.2 Å) and ~590 (5.7 Å)

Table 2

The Flexibility of Critical catalytic traid amino acid residues of Artherobactor, Arabidopsis and Human

S.No.	ENGase orthologs	Flexibility at catalytic triad	Gate keepers	Key residue for Transglycosylation residue
1	Atherobactor	N172, E173 and Y205 0.8 Å	W216 0.8 Å	F243 0.8 Å
2	Arabidopsis	N177, E179 and Y213 1.5 Å	N104 0.8 Å	Y243 2.4 Å
3	Human	N238,E240 and Y274 1.6Å	N164 1.6Å	Y304 2.4 Å

DISCUSSION

To understand the protein structure –functional relationship at molecular level. In this study we explored the similarity and variations of three different ENGase proteins (Athreobactor, Human and Arabidopsis) through molecular dynamics recorded upto 10ns. The resulted simulation event analysis gave RMSD, RMSF and secondary structural elements graphs. Based on the RMSD graphs, all three structures are considered as stable structures.The overall comparison of RMSF Graphs clearly indicates that when compared to bacterial ENGase plant and Human ENGases showed high flexibility all along protein structures and they were fluctuated around 0.8 Å to 6.4 Å and in case of Artherobacter the flexibility is only observed in C-terminal region near~585, which fluctuated around 3.6 Å at domain-3 and moderate flexibility of 2.1 Å is also observed near ~290. The flexibilities of three domains of three proteins are shown in the Table-1. In Arabidopsis, all the three domains showed high flexibility and at N-terminal at ~ 10 residues fluctuated up to 5.6 Å. In Humans, C-terminal region of domain-3 showed comparatively high fluctuations when compared to that of other two domains. As the active site of ENGase is present in Domain-1 region in all the three proteins, the flexibility of the catalytic residues of three orthologs was tabulated in Table-2. By comparing flexibilities of catalytic residues and observing the RMSF plots of all three proteins, the conserved catalytic triad of Arabidopsis and Human has shown good flexibility than Artherobacter. From the experimental results, the F243 is the key residue for transglycosylation activity in Artherobacter and is showing less flexibility than that of Tyrosine (Arabidopsis Y243 and in Humans at Y304) residue at the same position where the fluctuation is seen around 2.4 Å . From the data obtained, this flexibility may imparts faster transglycosylation in human and plant ENGases than that of bacteria.This provides a potent clue regarding molecular evolution of ENGases. Further Molecular dynamics simulation approaches have also been extensively used to report the structural

consequences that retain the function of these three orthologous proteins. The flexibility difference was clearly observed in RMSD, RMSF plot which is further supported by a difference in SASA value in all the three structures. This may produce a major impact on the structural conformational changes in all the three proteins.

CONCLUSION

In our analysis, we examined the structural consequences of the three ENGase orthologs through molecular dynamics simulations. A change in crucial amino acids at catalytic domain of ENGase orthologs was observed. The Tryptophan of Artherobacter was replaced with ASN in Human and Arabidopsis at gate keepers position and the key residue, Tyrosine was present in the place of phenyalanine of bacteria, which is essential for transglycosylation activity. Beacause of the change of these amino acids, variations in the flexibility was observed in the protein structure and were mapped with the experimental results. The sequence variations among different ENGase ortholog proteins finally reflect on the catalytic activity. This could also be considered as a natural selection criteria of evolution for elevated rates of enzyme activity in higher phyla.

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