Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(4):1575-1580

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

A comparative analysis of functional & structural impact of N171A in endo-β-N-acetylglucosaminidases through computational approach

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ABSTRACT

Endo-β-N-acetylglucosaminidases (ENGases) are the key enzymes showing dual specificity with an ability to catalyze hydrolysis and transglycosylation reactions.These enzymes have became the focal point for researchers because of their potentiality for synthesis of glycopeptides. Recently, few important ENGase mutants of Artherobacter protophormiae were screened that affects the catalytic activity of ENGase. Out of various mutants, the N171A mutant is having the property to abolish the whole enzymatic activity, both hydrolytic and transglycosylation activity (Jie Yin.,et al, 2009). In this study, we compared mutant N171A with native protein(PDB 3FHQ) to understand the structural changes, especially the hydrogen bonding pattern at the active site region which is causing the loss of catalytic activity in the mutant. The mutation position at 171 was mapped in the 3FHQ the native protein structure and a mutant with replacement of amino acid residue from Aspargine to Alanine was modeled by using homology modeling and simulation up to 10ns was done by Scrodinger Molecular modeling software. The results obtained from this computational analysis are correlated to experimental data and it Provides the potential insights to understand the key residues for catalytic activity of Atherobacter ENGase of both native and mutant protein to evaluate the stability, conformational differences .

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

INTRODUCTION

Endo-β-N-acetylgucosaminidases (EC 3.2.1.96) (ENGases) are the key enzymes that have the ability to 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].This enzyme activity has been described in wide variety of animal cells[3-7] bacteria [8–10], fungi [11,12], plants [13,14] and animals [15,16]. Endo-β-N-acetylgucosaminidases are categorized into glycoside hydrolase families GH18 and GH85 based on the homology in protein sequence .In case of the enzyme from *Flavobacterium meningosepticum* (endo-F) [17] 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. For example, Endo-A [27] can transfer a high-mannose type oligosaccharide to monosaccharides such as N-acetylglucosamine (GlcNAc) and glucose to form a new oligosaccharide [2, 18]. Transglycosylaation activity towards complex oligosaccharides is exhibited in ENGase from Mucor hiemalis (Endo-M) [19]. An intact oligosaccharide istransferred by Endo-A and Endo-M to the acceptor forms a new glycopeptide or glycoprotein in a single step, making it a highly convergent chemoenzymatic approach [20–22]. By using this chemoenzymatic approach a number of homogeneous natural and unnatural N-glycopeptides and glycoproteins are synthesized. [23, 24, 25].

From the recent reports of Takegawa *et al.* [26, 27] an endo-β-N-acetylglucosaminidase from *Aetherobacter protophormiae* (endo-A) has transglyccosylation activity, eventhough the hydrolytic activity still predominates [27]. The enzyme was also used for synthesis of neoglycoprotein by the process of transglycosylation[28].It was found that the transglycosylation activity can be enhanced by suppressing the hydrolytic activity of endo-A. Endo-A is specific for high-mannose or hybrid type N-glycans and has been applied for the synthesis of high-mannose type oligosaccharides and N-glycopeptides[29-31].

Although there is a considarable progress in preparation of proteins, synthesis of glycoproteins of defined structure is still a alarming challenge, especially with respect to the synthesis of carbohydrates to proteins [32] .From the recent findings it has been proved that synthetic oligosaccharide oxazolines (the mimics of the oxazolinium ion intermediate of the enzymatic reaction) can be used for Endo-A catalyzed transglycosylation. This approach has opened gates for expanding the scope of chemoenzymatic method for glycopeptides and glycoprotein synthesis [33]. 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 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 [34-36].

Previous experimental studies have provided an evidence that the mutant N171A is having the property to abolish the whole enzymatic activity, both hydrolytic and transglycosylation activity[37]. Furthermore the conformational change in the 3D structure of the protein accounts the changes in its time dependent physiological affinities towards the substrate and in turn affects the enzymatic function of ENGase. Here we have used the set of computational platforms homology-based structure modeling and molecular dynamics simulation studies which helps to understand the conformational changes of Artherobacter ENGase structure.

EXPERIMENTAL SECTION

Protein preparation:-

ENGase of *Artherobacter protophormiae* preparation was done by using protein preparation Wizard of Maestro software [38]. The protein was 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 [40].

Modeling and Preparation of insilico Mutant

This study focuses on the elucidation of 3D structure of Endo-A Mutant at 171 position by mutating Aspargine to Alanine and is done by homology modeling approach based on 3FHQ obtained from protein databank (PDB database).The Schrodinger molecular modeling software suite was used to build and refine the models.

Molecular Dynamics

Desmond Molecular Dynamics system [39,40,42] with Optimized Potentials for Liquid Simulations (OPLS) all-atom force field 2005 [41,43] was used to perform MD simulations of modeled mutant and native form, which were prepared using protein preparation wizard of Maestro interface [40]. Prepared structures were then uploaded to Desmond set up wizard for MD simulations. The protein structure is 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 counterions. 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 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 were 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 simulations trajectory with reference to their respective first frames. RMSF analyses was carried out for all the frames of 10ns MD simulation of ENGase native and mutant structures.

RESULTS AND DISCUSSION

N171A mutant:-

Asn(N)171 residue is a critical catalytic residue involved in bonding of Hydrogen with substrate present in beta barrel of domain1 main chain of functional ENGase of *Artherobacter protophormiae*. N171 is hydrogen bonded to the thiazoline nitrogen of substrate mimicking the ability of the asparagine to orient the acetamido group for a nucleophilic attack on the anomeric carbon[26] The oxazoline ion intermediate alternatively synthesizes via a substrate assisted catalytic mechanism. The side chain oxygen of N171 forms a hydrogen bond with the nitrogen of the C2-acetamido group of the substrate at a distance of 2.6 Å. Such an intramolecular attack can be avoided by mutating N171 to a non-nucleophilic residue and would provide an opportunity to develop glycosynthase-like ENGases. From previous experimental studies it was observed that N171A mutation of Endo-A abolished hydrolysis activity[40]. In silico mutations of Asn to Ala led to overall change in architecture of active site pocket. Slightly higher RMSD value was observed for mutant N171A compared to Native structure.

Docking with Substrate:

The native structure ENGase of *Artherobacter protophormiae 3FHQ [20] is taken from pdb database and* this structure is cocrystalized with NGT (N-glutamate thiozoline) this complex was prepared for simulations. The mutant N171A was mapped in the native structure using homology modeling module of schrodinger software. The energy minimizations were performed by OPALS force field for both the native structure and the mutant modeled structures (Fig. 1.) Further, to understand the structural changes in this mutant it was docked with substrate NGT . The ligand interaction plots of both native and mutant was shown in the figure-2. The native structure ligand-protein contacts shows that Asn171 is involved in H-bonding with substrate and the other amino acid residues like Tyr299, Tyr205, Trp93 and Phe169 at active site shows hydrophobic interactions with substrate and active site contains three water molecules involved in H-bonding. As the Asn171 is mutated with Ala171 the Hydrogen bonds are not formed in the mutant shown in (fig 2.B). The amino acid residues like Ala171, Trp93, Phe169, Phe243, Tyr299 at active site shows hydrophobic interactions with substrate and five water molecules were involved in H-bonding with substrate at active site. The number of water molecules increased in active site of mutant indicating that the solvent accessibility is increased when compared to that of the native structure. This might be one of the cause for the loss of functional activity in case of mutant

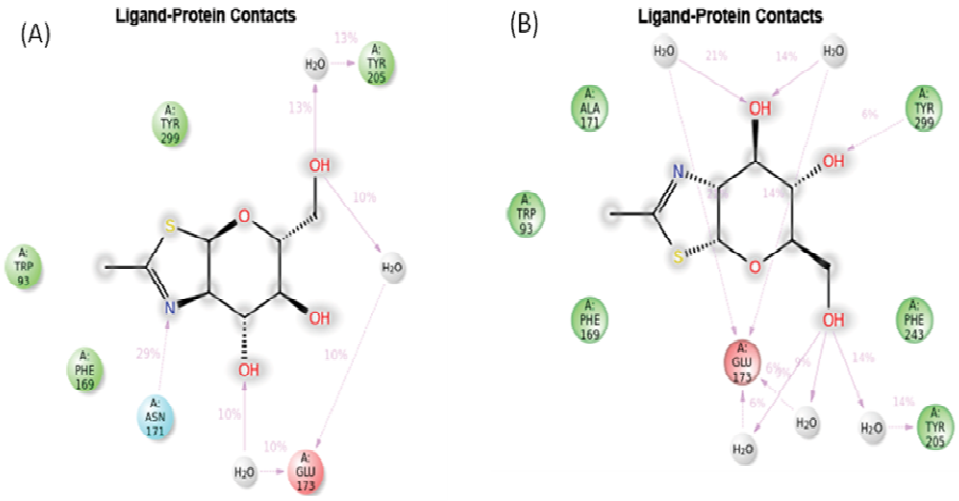

Fig: (A) Ligand-Protein contacts of Native structures **(B) Ligand-Protein contacts of** Mutant structures

Molecular dynamics simulations

To analyze the stability of predicted protein, RMSD of its backbone was plotted against the time dependant function of MD simulations [Figure-3]. Fluctuation in backbone of modeled protein during the simulations was recorded up to 10ns. After 5ns of MD simulations, backbone was found to fluctuate around 2.2 Å which persisted till the end of 10ns simulation. The standard deviation (SD) in RMSD for whole simulation process was found comparatively higher than 0.3Å for the last 10 ns of simulation time. These data suggest 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. A low RMSD throughout the MD simulation and consistent RMSD at the last of MD simulation indicated that the predicted tertiary structure of native had acquired a stable folding conformation. There is no much of deviation seen in RMSD between native and mutant of c-alpha but ligand interaction shows grater deviation between mutant and native structure as represented in the figure-3.There were lot of fluctuations seen in Lig fit Plot of mutant than in native. These results confirmed that mutant loss its stability.

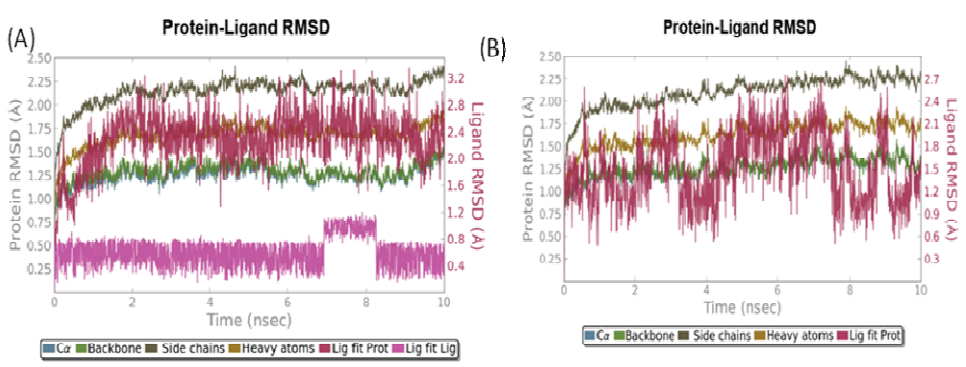

Fig3: (A) Time evolution of backbone, C-alpha, Side chains, Heavy atoms, lig fit prot. and lig fit lig RMSDs is shown as a function of time of the Native structures at 10000 ps.; **(B)** Time evolution of backbone, C-alpha, Side chains, Heavy atoms, lig fit prot. and lig fit lig RMSDs is shown as a function of time of the mutant structures at 10000 ps.;

Root Mean Square Fluctuation (RMSF) of all the residues was calculated during the 10ns MD simulations which was used to located the higher flexibility regions of the protein. The RMSF graph of native and mutant, most of the frames showed least flexibility variation and we observed high flexibility between 400-500 in mutant and 250-300 in native protein. These regions of higher variability belong to loop and secondary structure of ENGase protein. It is a well known fact that loop region tends to be more flexible than other part of protein. During the 10 ns MD simulations of Native ENgase protein, a separate analysis was done to calculate the deviation in only the loop regions of protein which revealed that these are the regions of higher flexibility of around 3.2 Å.

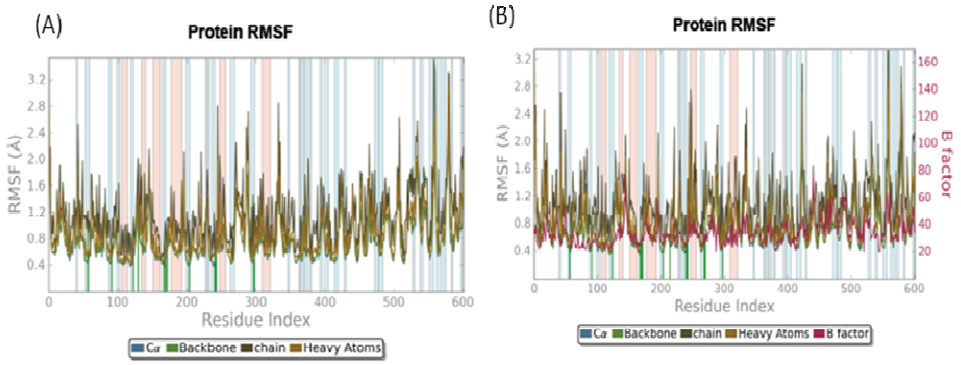

Fig 4 (A) RMSF of the backbone carbon alpha, Backbone, chain and heavy atoms over the entire simulation. The ordinate is RMSF (Ang) of Native; **(B)** RMSF of the backbone carbon alpha, Backbone, chain and heavy atoms over the entire simulation. The ordinate is RMSF (Ang) of Mutant;

CONCLUSION

Impact of single amino acid substitution on protein stability remains one of the most promising setbacks in protein science. This can be validated both experimentally and computationally, and offers new hope for a solution in the years ahead. In our analysis, we took the mutatant (N171A) of *Artherobacter protophormiae* ENGase which affects the functional activity. The mutations N171A was screened for its deleterious impact on protein function based on computational tools. To examine the structural consequences of these mutations, molecular dynamics simulations were carried out. A clear insight of stability loss of N171A mutation was observed in RMSD, RMSF and number of hydrogen bond when compared to other mutations. The results obtained from this computational analysis are correlated to experimental data and it provides the potential insights to understand the key residues for catalytic activity of ENGases not only in Atherobacter but also in other related orthologous species like Human & plant ENGases.

Acknowledgement
We are very thankful to the management of KL University, Vaddeswaram, Guntur District for providing facilities for doing this work.

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