Informatics in Biology, Health, and Food

Interdisciplinary Explorations in Life Science

Volume 2. Number 2. Dec. 2025 DOI: 10.22034/ibhf.2025.559174.1046

Original Article

In silico study on the effects of Glucosamine group on Thermal Stability of *Yarrowia lipolytica* Lip2 lipase

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Received: 2025/11/14 **Accepted:** 2025/12/16 **Online Published:** 2025/12/16

Abstract

Yarrowia lipolytica is recognized as a promising host for heterologous protein production due to its high secretion capacity. Among its enzymes, lipase plays a crucial role in diverse industrial applications, including detergent, food, cosmetic, pharmaceutical, and environmental sectors. Lip2, the only extracellular lipase of *Y. lipolytica*, is a glycosylated enzyme composed of 301 amino acids and several disulfide bonds. Its crystal structure reveals two N-glycosylation sites located at N113 and N134. Lip2 exhibits catalytic activity at low temperatures (around 5 °C), shows optimal performance at 37 °C, and rapidly loses activity above 50 °C. To explore the influence of sugar moieties on the thermodynamic stability of Lip2, the complete glycosylated structure was examined through molecular dynamics (MD) simulations. The simulations were carried out using GROMACS 5.1.4 with the CHARMM36M force field at three functional temperatures (300 K, 310 K, and 333 K) for a total production run of 40 ns. The results demonstrated that the glycosylated Lip2 exhibited higher temperature-dependent structural fluctuations, while the deglycosylated form showed enhanced thermostability upon temperature increase. Further analysis revealed that glycosylation affects not only the residues adjacent to the glycosylation sites but also induces conformational changes in distant regions of the protein. This leads to increased residue flexibility and a higher radius of gyration, which was supported by root-mean-square deviation (RMSD) analysis. Additionally, hydrogen bond analysis indicated that the non-glycosylated form maintained a higher number and longer lifetimes of hydrogen bonds across all temperatures, suggesting a more stable folding pattern. In conclusion, the overall stability of the glycosylated Lip2 decreases compared to its non-glycosylated counterpart, potentially due to complex molecular interactions. This in silico study provides new insights into the thermodynamic behavior of Lip2 in aqueous environments and establishes a foundation for future studies on the independent effects of glucosamine groups on enzyme stability.

Keywords: *Yarrowia lipolytica*; Lip2 lipase; Glycosylation; Thermal stability.

Cite this article: Esmaeili S., Gharouni M., Mosaddeghi H. In silico study on the effects of Glucosamine group on Thermal Stability of *Yarrowia lipolytica* Lip2 lipase. *Informatics in Biology, Health, and Food*. 2025;2(2):120-131.

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Introduction

The multifunctional nature of lipases has made them promising biocatalysts that have attracted significant attention in recent years due to their diverse functionalities. Among them, Lip2, a molecularly engineered extracellular lipase secreted by the yeast *Yarrowia lipolytica*, stands out. Owing to its high resistance to acidic pH and detergents, *Yarrowia lipolytica* Lip2 is used for a wide range of industrial, environmental, and nutraceutical applications.

This protein consists of 334 amino acids encoded by the *Lip2* gene, including the first 33 residues that form a signal peptide (or pre-protein sequence), followed by a mature glycosylated protein of 301 amino acids (1). Lip2 is an extracellular lipase characterized by several disulfide bonds, a classical α/β-hydrolase fold, and four disulfide bridges (2). It contains six α-helices and eight β-sheets (3). The crystal structure reveals two glycosylation sites at N113 and N134 (4,5).

Another important structural feature of lipases is the lid domain, a mobile element formed by residues Thr88 to Leu105. This single α-helix covers the active site and undergoes a conformational change at the lipid–water interface, thereby exposing the catalytic site to the substrate. The catalytic triad of Lip2 consists of Ser162, located in the nucleophilic elbow after the β5 sheet, Asp230, and His289, which are found after the β7 and β8 sheets, respectively. The typical GxSxG motif of lipases is represented by the GHSLG sequence in Lip2 (2).

Glycosylation of this protein was first identified in 2007. The N113 site (loop M101–H126) and the N134 site (helix α3) are the two glycosylation sites found in Lip2 (6). The two sugar moieties attached to the asparagine residues N113 and N134 correspond to two different high-mannose structures, $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$, respectively (13). The first glycosylation site participates in several intracellular interactions, including five polar interactions with residues D145 (helix α3), D174 (helix α4), N178 (helix α4), and H180 (loop V177–L183). In contrast, the N-acetylglucosamine attached to N134 interacts with only one or two polar contacts, including L110, depending on the chain considered in Lip2 (6).

Evidence suggests that Lip2 has low thermal stability (7). It is active at low temperatures (around 5 °C) and rapidly deactivated above 50 °C, with maximum activity at pH 7 between 25–55 °C and an optimum at 30–40 °C (8). Residual activity measurements at 25 °C, 37 °C, and 60 °C indicate half-lives of 156.5 h, 106.8 h, and 0.058 h, respectively (9). Moreover, it loses hydrolytic activity after 15 minutes of incubation at 60 °C, while remaining moderately stable at lower temperatures. Interestingly, immobilized Lip2 has been used in biopolymer synthesis at temperatures up to 150 °C (10). In this study, we examine the effect of glycan moieties on the thermal instability of this protein.

The *Y. lipolytica* lipase Lip2 (YlLip2) has numerous practical applications, such as in cheese maturation and dry fermented sausages (11), kinetic resolution of racemic compounds (2), functional ester synthesis (12), biodiesel production (13,14), wastewater treatment, and enzyme replacement therapy for pancreatic enzyme insufficiency (15). For example, immobilized YlLip2 has been shown in Chinese experiments to convert plant, animal, or used cooking oils with high free fatty acid and water content into fatty acid alkyl esters, achieving over 90% conversion at 40–50 °C (16). Moreover, heterologous expression of YlLip2 in *Pichia pastoris* has resulted in an exceptionally high production level of 42,900 U/mL, demonstrating its industrial potential (17). These properties highlight Lip2 as a highly efficient lipase enzyme with remarkable extraction and conversion capabilities (18).

Patients suffering from pancreatic exocrine insufficiency (PEI)—primarily associated with chronic pancreatitis or cystic fibrosis—experience impaired digestion and malnutrition due to insufficient pancreatic enzyme secretion (6,19). The current treatment involves oral administration of porcine pancreatic extracts (pancreatin) containing lipase, proteases, and amylase (20). However, animal-derived pharmaceutical products carry potential risks of viral transmission. Moreover, to prevent enzyme degradation in the stomach, they require microgranule coatings for protection before

reaching the small intestine. Therefore, there is a need for novel recombinant enzymes with high activity and improved stability under gastrointestinal (GI) tract conditions. Among these, lipases are critical because of their essential role in fat digestion and energy acquisition. Microbial lipases are attractive candidates for pancreatic enzyme replacement therapy (PERT), as they are already produced on an industrial scale (4), and could yield better therapeutic results than current pancreatic extracts (21,22).

Although most microbial lipases are inactivated by gastric acidity or bile salts (23,24) and degraded by digestive proteases, YlLip2 exhibits high activity toward long-chain triglycerides (TAGs) and maintains stability at low pH levels similar to those in the stomach and intestines of PEI patients. Indeed, YlLip2 is more active than acid-stable dog gastric lipase (DGL) at pH 4.0 (24,25). Nevertheless, like most mesophilic enzymes, YlLip2 suffers from low thermal stability, limiting its industrial applications (22,26).

Glycosylation, the covalent attachment of complex polysaccharide chains (glycans) to proteins, is a common post-translational modification that significantly affects protein structure and dynamics. Glycans influence enzyme activity, protein–protein interactions, and the circulation half-life of therapeutic proteins (35). In eukaryotic cells, glycosylation begins during protein synthesis in the ribosome at the endoplasmic reticulum surface, where glycans are added to unfolded proteins during translocation, aiding in proper folding (28). The attachment of hydrophilic carbohydrate moieties effectively modulates protein properties by introducing structural diversity. Glycans also function as recognition markers in protein trafficking (29), mediate host–pathogen interactions, regulate immune responses, and modulate enzyme activity and turnover (30,31). They can alter structural, kinetic, and thermodynamic features of proteins, adding an additional layer of information beyond the amino acid sequence (32).

Despite progress in glycopeptide and carbohydrate synthesis, the relationship between glycans and protein folding kinetics or stability remains unclear (33). Statistical analyses of glycan-containing structures in the Protein Data Bank (PDB) reveal that approximately 6% of all entries contain at least one glycan chain. Of these, about 56% are N-glycosylated, 4.3% are O-glycosylated, and 39% contain noncovalent ligands (34). Glycosylation directly affects protein structure, functionality, and stability, influencing both folding and native-state conformations (35). Understanding which glycans are critical for function or recognition remains challenging. Thus, studying the structure and dynamics of saccharide–protein complexes is crucial.

Recent advances in computational biology have made such studies increasingly feasible. The GROMACS molecular dynamics package now allows efficient setup and simulation of glycoproteins, simplifying research on glycosylation effects (36). Carbohydrate modeling using CHARMM force fields has also become a standard tool for exploring protein–carbohydrate interactions (37,38). Nevertheless, the structural complexity of glycans increases the susceptibility to modeling errors (39).

The extracellular lipase from *Y. lipolytica* (YlLip2) is a glycoprotein containing approximately 12% carbohydrate by weight. In this study, we investigated the effect of glycosylation on the thermal stability of YlLip2 through in silico molecular dynamics simulations at 300 K, 310 K, and 333 K. We also analyzed RMSD, RMSF, and Rg plots for both glycosylated and non-glycosylated forms of the enzyme.

Methods

Starting Structures

To analyze the impact of sugar moieties, the structures of the lipase with and without glycans were required. In this study, we used the non-glycosylated mutant form (N113Q/N134Q) with PDB ID 4JEI, and the wild-type structure with PDB ID 3O0D of *YlLIP2*, both available in the RCSB Protein Data Bank.

Molecular Dynamics Simulation Procedure To provide a comprehensive explanation of the chemical and thermal stability and to accurately evaluate the effects of sugars, the entire protein structure, including its glycoside groups, was modeled. A meaningful comparison was then performed between this glycosylated structure and the recombinant non-glycosylated form of *Lip2* lipase, as suggested by the Aloulou research group.

Molecular dynamics (MD) simulations were carried out using GROMACS 5.1.4 (44) with the CHARMM36M force field. Simulations were conducted at three functional temperatures (300 K, 310 K, and 333 K) for 40 ns of production time. The Verlet cutoff scheme was employed for all short-range non-bonded interactions, with the real-space contribution to Coulombic terms truncated at 1.2 nm, and short-range van der Waals interactions also truncated at 1.2 nm.

All bond lengths were constrained using the LINCS algorithm (45), allowing a 2-fs integration time step. The neighbor list was updated every five simulation steps (10 fs). Long-range electrostatic interactions were computed using the Particle Mesh Ewald (PME) method (46).

According to previous studies on glycoprotein simulations, the Berendsen thermostat is inadequate for such systems. Therefore, we applied the Nose–Hoover thermostat to maintain the simulation temperature (37,38). The trajectories were analyzed using the GROMACS analysis tools (47), and thermodynamic folding parameters for both protein forms were visualized using Grace software (48). The Parrinello–Rahman barostat (49) was used to control pressure during equilibration and production simulations under the NPT ensemble.

Results

To better understand the effect of glycan attachment on the structure and dynamics of *Yarrowia lipolytica* LIP2 lipase (YlLIP2 lipase), two forms of the protein—one glycosylated and one non-glycosylated at specific sites—were subjected to molecular dynamics simulations. To investigate the impact of N-glycosylation on the thermal stability of the protein, six independent simulation runs were performed at three different temperatures for both the non-glycosylated and glycosylated forms of the lipase.

In this regard, we examined the influence of N-glycosylation on both the local and global structures of the protein by comparing the structural similarity between the glycosylated and deglycosylated forms of YlLIP2 lipase at various temperatures. Comparative structural analyses indicated that glycosylation does not significantly affect the overall protein conformation.

The root-mean-square deviation (RMSD) was plotted to assess the stability of each protein or glycoprotein system at different temperatures relative to their initial structures. Based on the definition of RMSD, this parameter reflects the global structural similarity to the original protein structure obtained from the RCSB database. Thus, RMSD serves as an indicator of protein folding status. In other words, changes in RMSD distribution provide valuable insight into the extent of structural fluctuations. Generally, the RMSD value should not exceed 2 Å during simulations.

Accordingly, the overall slope of the RMSD plot represents the structural stability of the protein during molecular dynamics simulations—the closer the slope is to zero, the more stable the protein is. The results suggest that glycan groups do not behave consistently across the three temperatures tested. Glycosylation significantly increases RMSD at higher temperatures (310 and 333 K), whereas the protein non-glycosylated exhibits a more stable structure with a decreasing RMSD trend.

Furthermore, for industrial applications such as detergent production, the GROMACS simulation results at 333 K indicate worsening RMSD behavior for the wild-type (glycosylated) protein, with a slightly positive slope. At the end of the simulation, the plateau region of the RMSD curve for the glycosylated form lies above that of the non-glycosylated protein, illustrating greater structural dynamics and an increased probability of adopting defective conformational states, which leads to lower overall stability (Figure. 1).

At different temperatures, the sugar-bound and sugar-free states were analyzed and compared separately. The results indicated that, at 300 K, the sugar-bound lipase exhibited smaller fluctuations and a more stable structure compared to the sugar-free form. At temperatures of 310 and 333 K, from 30 ns until the end of the simulation, both the sugar-bound and sugar-free states showed comparable stability (Figure. 2).

Moreover, to investigate the effect of N-glycosylation on residual flexibility, the root-mean-square fluctuations (RMSF) were

analyzed. Further evaluation of both local and global structural features was performed to determine the impact of N-glycans on the lipase structure.

Except for the glycosylation sites themselves, an overall increase in RMSF values was observed in comparison with the deglycosylated form. The glycans were attached at Asn113 and Asn134, while the residue His217, which exhibited the largest dynamic fluctuations and RMSF differences upon glycosylation, was not located directly at a glycosylation site.

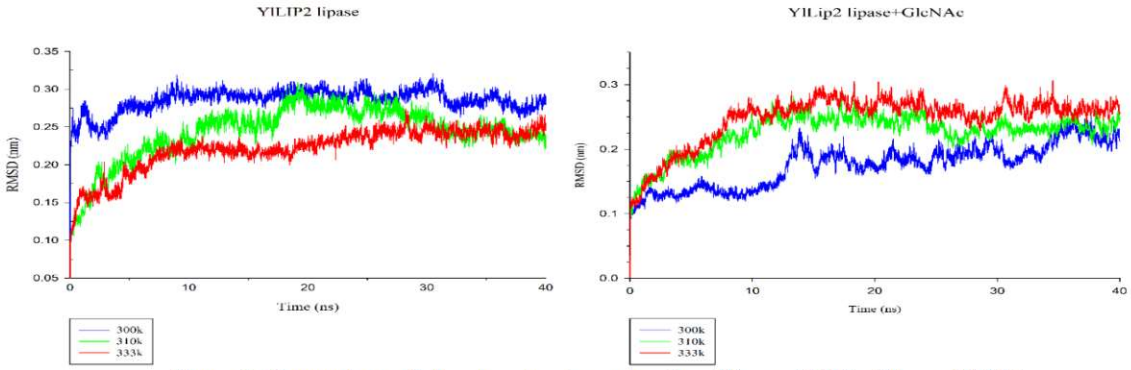

Figure 1. Comparison of glycolysed and nonglycolysed form of YlLip2 lipase RMSD.

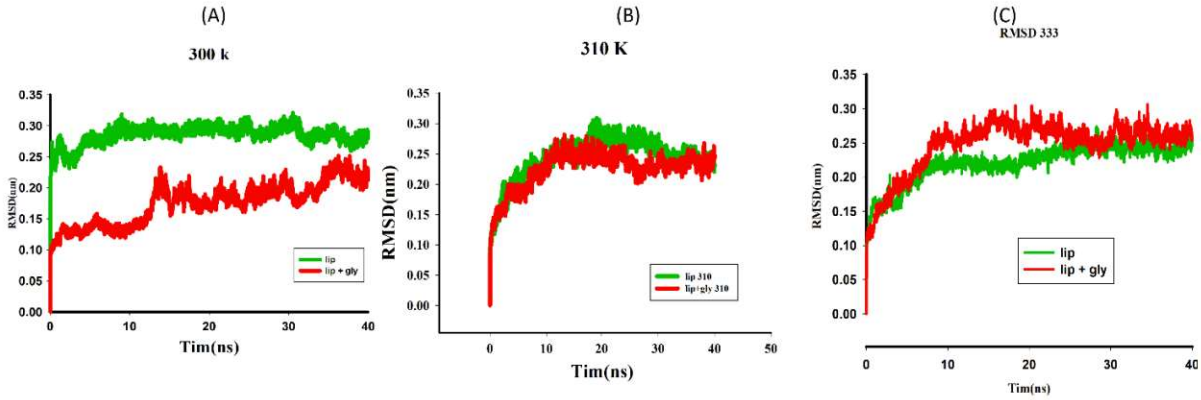

Figure 2. Comparison of glycolysed and nonglycolysed form of YlLip2 lipase RMSD at different temperature.

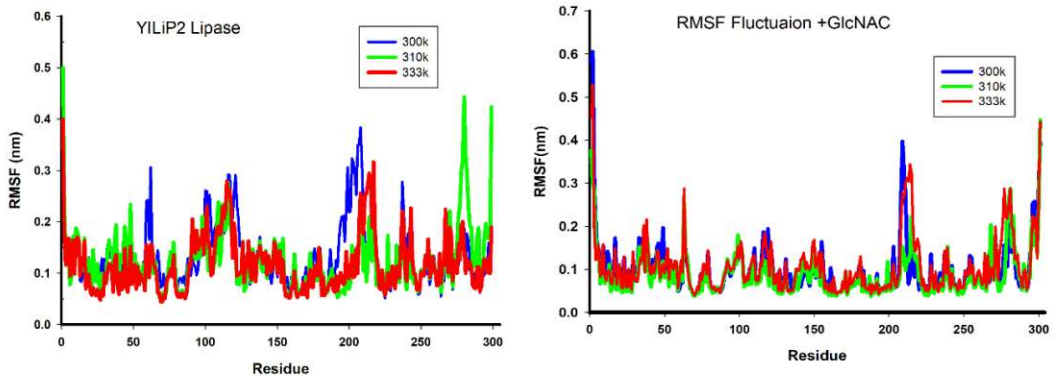

Figure 3- Glycosylated and non-glycosylated form of YlLip2 lipase RMSF.

In addition, the RMSF plots revealed that residues with inherently high instability and maximal fluctuations, such as His217, experienced more pronounced conformational changes in the glycosylated protein than in its deglycosylated counterpart (Figure.3).

Based on the temperature-dependent variations, the amino acids to which the sugar is attached—namely Asparagine 113 and Asparagine 134—were identified. The results indicate that at 310 K, the amino acids across the overall protein structure exhibit greater stability (Figure.4).

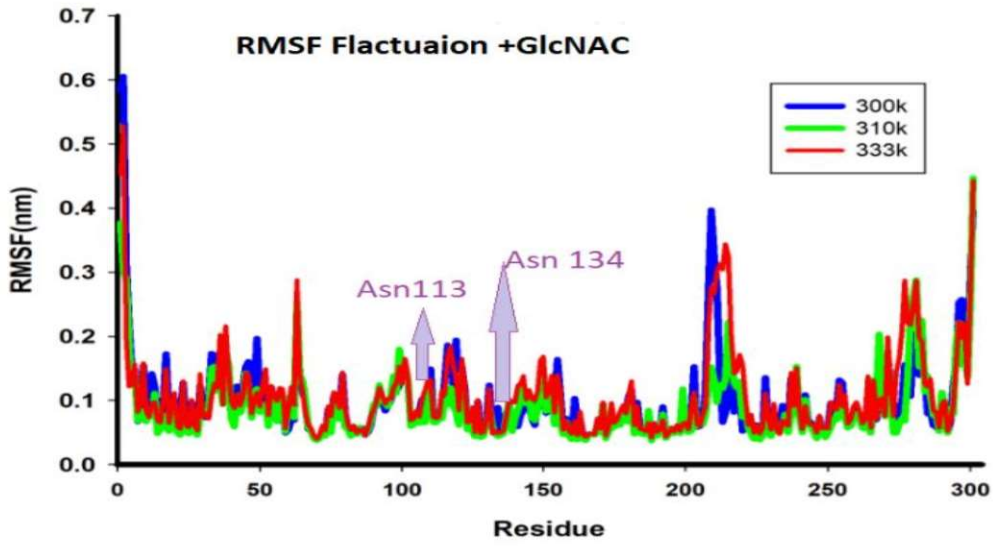

Figure 4. Illustrates the fluctuations of amino acids in the sugar-bound state of the protein at different temperatures.

One of the analyses performed in this study focused on the amino acids and the types of interactions formed between them and the sugar present in the glycosylated protein structure. Given that this protein contains two sugars attached via glycosidic bonds at the Asparagine 113 and Asparagine 134 positions, the results at 300 K showed that the sugar linked to Asparagine 113 forms four hydrogen bonds with Asparagine 113, Aspartic acid 145, and Asparagine 174. In addition, this sugar establishes van der Waals interactions with Glycine 141 and Asparagine 148. In contrast, the sugar attached to Asparagine 134 forms only one hydrogen bond with Leucine 110, while the remaining interactions are of the van der Waals type, involving Alanine 112, Asparagine 134, Asparagine 127, and Isoleucine 130 (Figure 5).

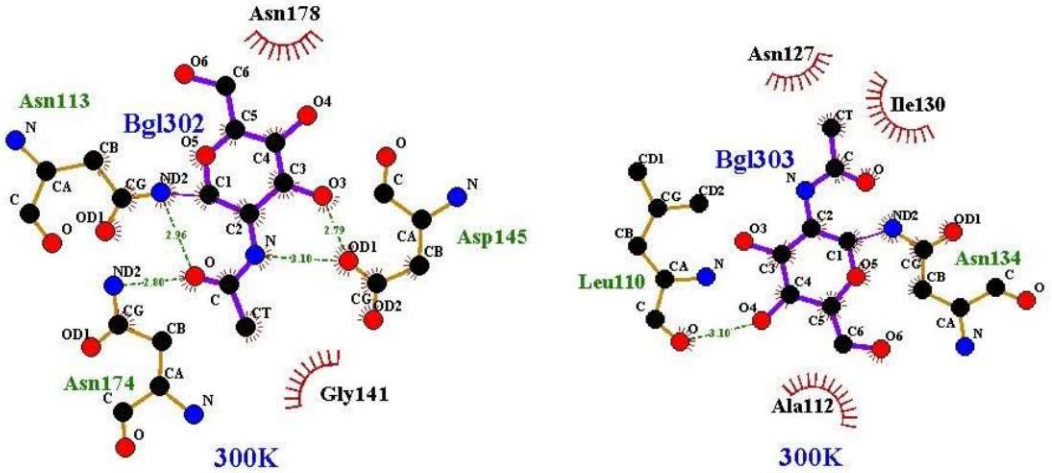

Figure 5. Amino acids involved in interactions with the sugars present in the glycosylated protein at 300 K.

At 310 K, it was observed that Asparagine 113, in addition to the glycosidic bond, forms one hydrogen bond with the sugar, while another hydrogen bond is established with Asparagine 174. The remaining interactions are of the van der Waals type and involve Asparagine 178 and Aspartic acid 145. Furthermore, the sugar located at the Asparagine 134 site forms van der Waals interactions with Leucine 110, Asparagine 127, Isoleucine 130, and Glutamine 131 (Figure 6).

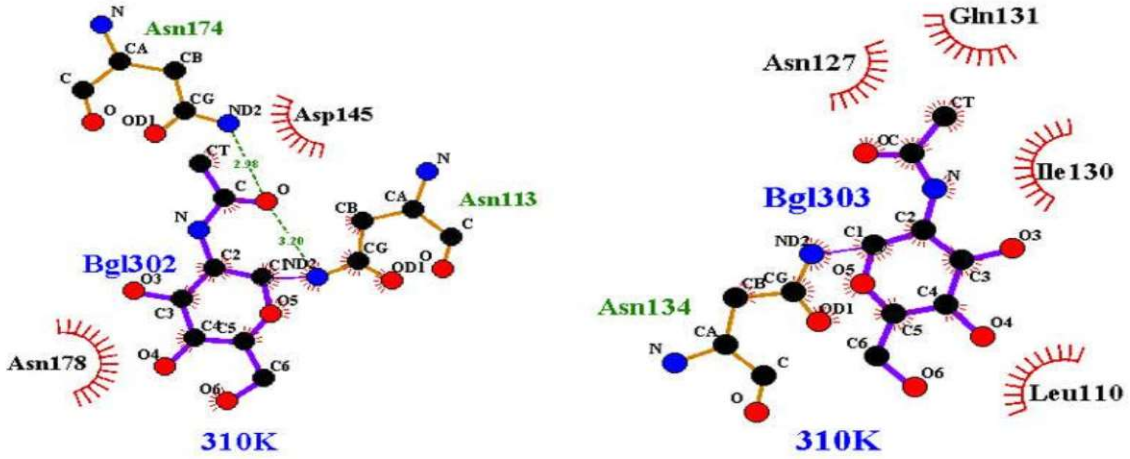

Figure 6. Amino acids involved in interactions with the sugars present in the glycosylated protein at 310 K.

At 333 K, the sugar attached to Asparagine 113 forms only a single hydrogen bond with Histidine 180, while the remaining interactions with Phenylalanine 171, Leucine 175, and Asparagine 178 are of the van der Waals type. At the Asparagine 134 site, all interactions with Leucine 110, Alanine 112, Asparagine 127, Isoleucine 130, and Glutamine 131 are exclusively van der Waals interactions (Figure 7).

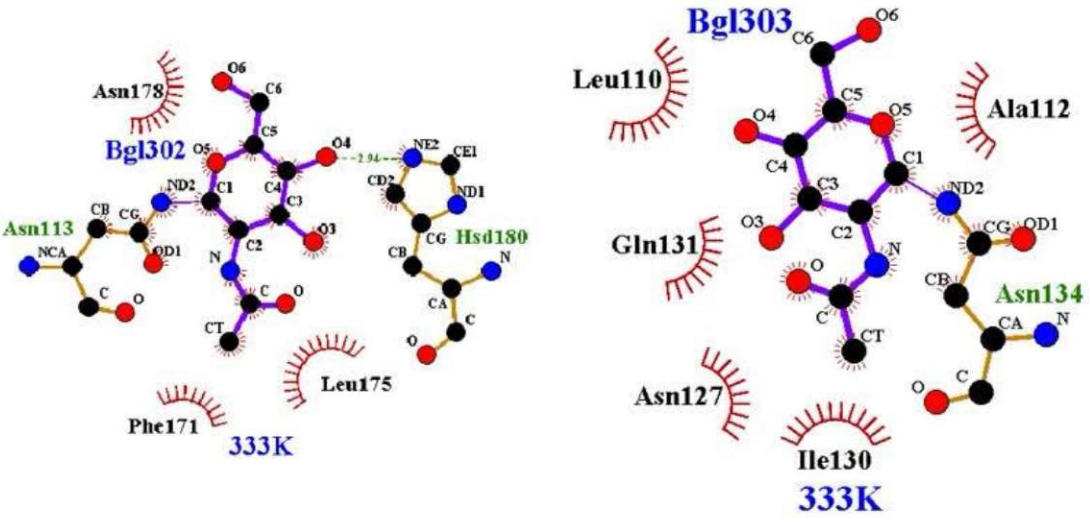

Figure 7. Amino acids involved in interactions with the sugars present in the glycosylated protein at 333 K.

Ultimately, the RMSF plots at 310 K showed the lowest fluctuations in both glycosylated and non-glycosylated forms. In contrast, the protein exhibited the highest instability at 300 K for the glycosylated form and at 333 K for the non-glycosylated form. The overall stability of the glycosylated structure also decreased with increasing temperature (Figure 2).

From these observations, we concluded that glycosylation does not only influence the localized residues near the glycosylation sites, but its effects are also propagated to other regions of the protein. To visualize the superimposition of RMSF values on the three-dimensional structure, the β-factor representation was employed (Figure 8).

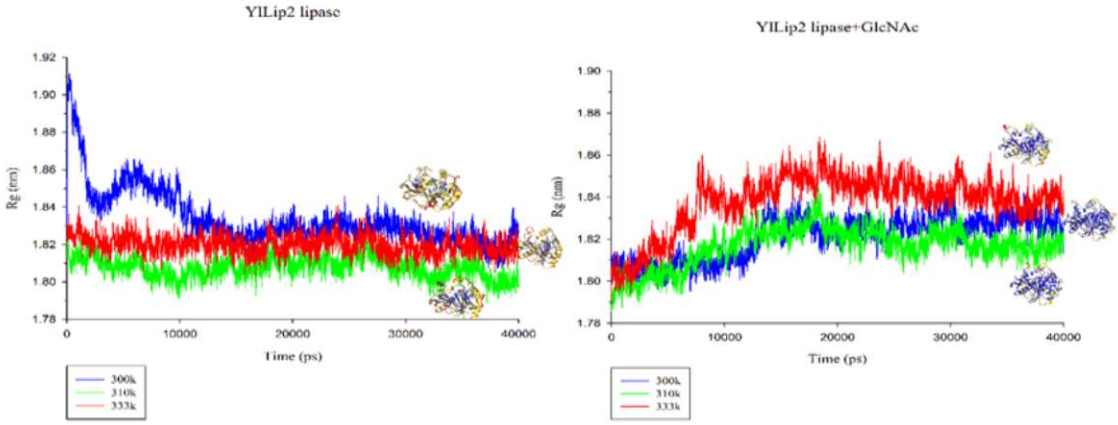

Figure 8. Radious of gyration and flexibility of structure.

The spiral plots of the glycosylated and deglycosylated states show that glycosylation promotes correct protein folding and thus leads to improved protein performance, which is consistent with the simulation results at 300 K (Figure 9). Additionally, the radius of gyration (Rg) was calculated to determine the density of the protein structure. Rg defines the root-mean-square distance of protein atoms from the center of mass, which here refers to the Cα atom of the protein.

Based on the relevant data, the quantified Rg of the glycosylated form of the protein showed greater expansion at higher temperatures, whereas the deglycosylated form of *YlLip2* lipase became more compact at 310 K and 333 K compared to 300 K. It is known that modifications in protein volume during simulations at different temperatures are caused by a range of factors.

Defining indices for the protein–glycan system helps to identify intra- and inter-structural hydrogen bonds between protein and sugar moieties. It was revealed that in the glycosylated form of the lipase, the number of hydrogen bonds did not correlate directly with the Rg results.

The calculation of salt bridges followed an inverse relationship with the protein's radius of gyration, except for the glycosylated protein at 333 K. At 333 K, despite an increase in Rg, a noticeable rise in the number of salt bridges was observed from 8 at 310 K to 14 at 333 K.

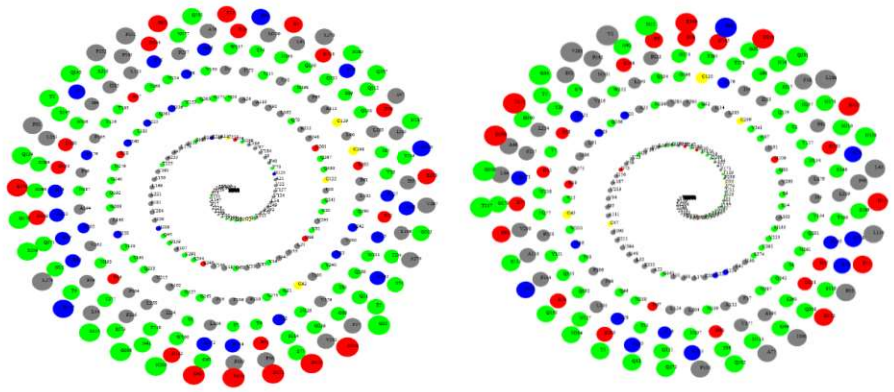

Figure 9. These spiral plots are based on sorting residues upon their relative solvent accessibility.

The radius of each sphere representing a residue is proportional to the solvent-accessible surface area of that residue. The inner residues in the spiral represent buried amino acids, whereas progressively more exposed residues are located closer to the outer ring of the spiral (50).

The protein maintains its folded structure due to several important salt bridges, hydrogen bonds, and hydrophobic interactions within the deglycosylated form. All of these data are in agreement with the results obtained from defining the secondary structure of the protein using the DSSP algorithm, which was implemented to assign the secondary structure of each amino acid during the simulation period. The secondary structure, given the atomic coordinates, geometrical features, and solvent exposure of the protein, was determined via DSSP.

Although it is a common mistake to believe that the algorithm predicts protein structure, the comparison of the secondary structures of the proteins with each other clarified that their native secondary structures were preserved. The main difference was observed for the N-terminal residues, whose β-bridge structures in the non-glycosylated protein at 300 K disappeared, while some partial α-helices were identified in the glycosylated lipase at 333 K.

Moreover, the structure of residues 99–126, located around the glycan sites, changed from turn and bend structures in the absence of glycans to more stable conformations such as α-helix and β-sheet in the glycosylated protein. This clearly indicates that glycans restrict structural changes around the glycosylation sites. However, a systematic analysis of the DSSP results supports the previous observation related to the instability of structures induced by N-glycosylation at higher temperatures.

Discussion

Yarrowia lipolytica Lip2 (YlLip2) lipase is a highly versatile catalyst, which has made it a well-known protein in diverse fields. Therefore, extensive research has been conducted on its structure to enhance thermal stability. Implementation of mutations and genetic engineering has been suggested as one of the strategies. Saturation mutagenesis libraries were created at rationally selected amino acid positions 103, 116, 117, 119, 121, and 239. A combination of these thermostabilizing mutations led to a thermostable YlLip2 variant with a 7-fold increased half-life of thermal inactivation compared to the wild-type lipase. To the best of our knowledge, this is the first report in which both a random mutagenesis-based strategy and the B-FIT method were implemented in parallel to improve lipase thermostability, demonstrating that the combination of these two methods is advantageous (16).

Based on a study assuming the therapeutic potential of YlLip2 lipase for enzyme replacement therapy in cases of pancreatic exocrine insufficiency, research into the efficacy of two-point mutations causing YlLip2 deglycosylation via the culture medium—considering wild-type and mutant forms with one or two glycosylation sites deleted—showed that glycosylation is not crucial for secretion or catalytic activity (13). In this context, critical properties for oral administration were investigated in both wild-type and non-glycosylated forms. It was observed that lipase specific activity and adsorption at the lipid–water interface decreased in the absence of N-glycosylation. Moreover, the glycosylated enzyme exhibited clearly better stability in the presence of pepsin and chymotrypsin in vitro (51).

However, no rational and persuasive explanation for the influence of glycans on YlLip2 lipase thermal stability was found in previous studies. Herein, we explored the impact of N-glycosylation on YlLip2 conformation, dynamics, and its role in modifying protein stability. Consistent with recent studies, our data show that glycosylation considerably reduces the protein's thermal stability. Glycosylation also affects kinetic activity and adsorption, while deglycosylation reduces the enzyme's hydrophilic properties.

Given that intramolecular electrostatic and hydrophobic interactions, as well as hydrogen bonds, are critical for protein folding, analyses of simulated protein structures are highly informative. The mutation N→Q induces replacement of asparagine with glutamine; both belong to the same amino acid group, and this mutation removes the sugar moiety from the protein.

RMS fluctuation plots indicate that the sugar-free protein exhibits greater fluctuations than the glycosylated protein at different temperatures.

The first unstable region is located in the β-strand and loop between residues 57–63. The presence of negatively charged amino acids creates repulsive forces, destabilizing the structure. Some of these residues also have higher α-helix propensity than β-strand or loop structures, contributing to instability in both forms.

The second unstable region spans residues 97–123. This region is more unstable in the deglycosylated form at 300 and 310 K, due to the presence of polar and similarly charged residues. The N113Q mutation, which removes the sugar moiety, is located in this region, further increasing instability at normal temperatures.

The third unstable region includes residues 193–217, with residue 207 showing the highest flexibility. Deglycosylation of N113 and N134 at 310 K clearly affects this region. At 333 K, the deglycosylated lipase shows a notable increase in RMSF, Rg, and RMSD compared to lower temperatures. Additional factors contributing to instability include the proximity of phenylalanine residues favoring α-helices, and glycine residues promoting loop formation. Finally, the last instability in the structure arises from residue tendencies to adopt alternative secondary structures and the effects of deglycosylation. Although the unstable ranges are similar in glycosylated and deglycosylated forms, the number of flexible residues is higher in the deglycosylated protein. The region 193–212 shows the greatest instability, with GLU209 being the most flexible residue due to its positioning adjacent to two polar residues that favor α-helix formation.

In agreement with previous studies on glycan impacts, glycosylated proteins show lower stability at higher temperatures. At elevated temperatures, the deglycosylated form exhibits more pronounced thermal destabilization. Since the amino acid sequences are identical in both forms and asparagine and glutamine belong to the same group, differences in stability are attributed to glycosylation.

Lu et al. (2012) studied a 46-β-barrel model protein with distinct glycosylation sites using molecular dynamics simulations. Their data indicated that more flexible and hydrophobic glycan sites improve protein stability, whereas glycans at highly buried residues decrease stability (52). However, MD simulations are inherently limited by their restricted time and length scales, high computational cost, and the approximate nature of classical force fields (53). Consistently, the lower-to-middle flexibility of N113 and N134 results in thermal instability when glycan chains are attached.

In conclusion, our findings suggest that omitting glycan moieties is a feasible approach to improve YlLip2 lipase thermal stability for industrial a aplications at higher temperatures.

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