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Mechanistic insights of glycosylation-dependent cell adhesion molecule-1 with L-selectin expressed in the lactating bovine mammary gland

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***Abstract*---**Most of the dairy cows often have immune suppression during calving time and leads several metabolic disorders and manifesting of poorly functioning immune system. Hence, the interaction between the immune system and metabolism of dairy cows is core important to read in terms of benefit to dairy industries. In this, the transition of dry period to lactation period is at most risk periods for the dairy cow's body mechanism and by this time, there are several changes occur in the protein level. There have been several studies focused on casein protein and its functions in the lactation mechanism in mammary glands. But there are few other proteins, which are contributing directly or indirectly in the lactation process, and it is compulsory to understand those contributions up to its structural level. In dairy cows, the Interactions between the L-selectin

and Glycam1 is core important protein -protein interactions, but the information's on these systems are lacking. This study aims to provide the mechanical insights of L-selectin binding over the Glycosylation-dependent cell adhesion molecule 1 (Glycam1) in *Bos taurus*. Through this work, we have represented the structural details of Glycam1 and L-selectin along with their interactions is visualized. Overall, we believe that this study will provide a way to future works on understanding the interactions between the immune system and metabolism of dairy cows.

***Keywords*---**glycam1, L-selectin, Bos Taurus, protein-protein docking, molecular dynamics.

Introduction

Dairy industry is the major growth sector with the turnover of 450 billion USD with gradual increase of Compound Annual Growth Rate (CAGR) of 5% per year and this provides high contribution to various countries Gross domestic product (GDP) growth [1]. But the production gap between supply and demand for milk is increasing by approximately 10% annually and therefore it is mandatory to understand the mechanisms regulating bovine milk synthesis in dairy cows [2, 3]. The recent developments in integrating the transcriptomic and proteome analysis guide the researchers to understand deep insights of molecular mechanisms driving biological process in cells and tissues [4, 5]. There are several mechanisms depends on several amino acid derived proteins for maintenance of vital functions, reproduction, growth, and lactation. Especially in the mechanism of lactation, the mammary gland requires huge number of amino acid involvements to synthesize milk proteins [6, 7]. This mechanism of amino acid processing to milk protein conversion in mammary gland is too complicated and it is mandatory to examine those reactions, interactions behind the mechanisms [8, 9]. Here, the mechanism is diverged to several amino acid conversions, oxidation, and reduction reaction mechanism for energy productions. In process of milk synthesis, the mammary glands utilize maximum amino acids and 30g of protein per kg of milk is a final product [10, 11].

Even though in this mechanism, most of the available studies have high lightly showing the contribution towards the casein protein, it is also mandatory to study the other proteins involved in this mechanism [12, 13]. In this regard, there is another class of proteins which also secreted as external secretion which is in the form of mucins or muscin-like proteins found in mammary gland [14, 15]. It has been reported in humans, the high molecular weight milk-mucin complex is formed due to aggregation of milk fat globules associated with mucin-like glycoprotein and are responsible for antiviral activity against rota-viruses [16, 17]. Glycosylation-dependent cell adhesion molecules 1 (GlyCAM-1) is an endothelial glycoprotein acts as adhesion molecules present in mammary glands functions as an endothelial cell surface ligand for leucocytes adhesion molecule L-selectin [18]. The family of selectin proteins comprises of three closely related cell–surface molecules: L, E and P–selectin, which are named after the cells in which they are expressed [19, 20]. From the literature information, there are few evidences

showing the details behind the Glycam-1 interactions with L-selectin in various organisms, but not in *Bos Taurus* [21]. Now it is mandatory to bring out those interaction details in atomic level to the scientific community. So that, here our objective is to elucidate the mechanical insights of L-selectin binding over the Glycosylation-dependent cell adhesion molecule 1 (Glycam1) in *Bos taurus*.

This biochemical signal transduction pathway mediates the selectin-mediated leukocyte activation in this organism. Common mechanism evidence shows that, the interaction with L-selectin molecules GlyCAM-1 needs to be sulfated especially on oligosaccharide side chains to bind to L-selectin During this process, both mono and disaccharides of GlyCAM-1 were sulfated as Gal-6-SO4, GlcNAc-6-SO4 and Galβ14(So4-6) GlcNAc [22]. The major capping structure of GlyCAM-1 contains there are three structural elements such as sialaic acid, fucose, and sulfate and the capping structure was determined as 6'-sulfated sialyl lewisX [23]. These two sulfated O-glycans such as 6'-sulfo-sLeX and 6-sulfo-sLeX, with So4 linked to position 6 of Gal and GlcNAc respectively were found in a core-2 structure can block the binding of L-selectin to GlyCAM-1 [24]. Proteolytic cleavage of L-selectin may inhibit leukocyte accumulation and appears to occur in various physiological contexts, including the inflammatory response [25]. L-Selectins contains cytoplasmic domain which has about 17 amino acids essential for normal function and interact with carbohydrate binding site of GlyCAM-1 especially of sialyl lewisX [26, 27]. Based on these available information's, we tried to bring up the mechanical insights of L-selectin and Glycam-1 Interactions using the modeling and interactions studies. As far as, there are only few studies reporting about this L-selectin and Glycam1, we tried to bring those structural information's through this work.

Materials and Methods

Sequence information

The sequence information of Glycam1 (ID: Q02596) and L-selectin (ID: P98131) from the organism *Bos Taurus* is taken from the uniport protein sequence database (www.uniprot.org) in the fasta format. In search of experimental structures, the protein data bank informs the lack of Glycam1 and L-selectin experimental structures for this organism. Followed by this, the template structures from relatively closer functional protein and organism is searched through the protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [28, 29]. This is performed to observe the template structure from relatively closer Glycam1 and L-selectin experimental structures in the protein data bank for modeling the 3D structures. The amino acid sequence of both Glycam1 and L-selectin were used as input and the option Psi-BLAST was selected to search for templates.

Protein structure Modeling

Sequence similarity indicates that, lack of suitable templates for Glycam1 and for L-selectin, the blast shows the template of L-selectin protein structure from *Homo sapiens* with 84% identity [30-32]. Lack of template structure for Glycam1 force to apply the threading method for this sequence and for L-selectin, the homology modeling is performed. The sequence of Glycam1 is to be designed via threading

method through I-Tasser (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) [32]. I-TASSER is tool based on threading algorithm, via hierarchical protocol for automated protein structure prediction and structure-based function annotation [33, 34]. For the L-selectin, the 3D structure is generated through academic version 9.21 of Modeller software (https://salilab.org/modeller/) [35, 36]. Based on the DOPE score value, the lowest optimized protein energy score model was selected for further quality analysis [37, 38].

Dynamic behavior of Model proteins

Both the proteins taken for the study is modeled via computational methods; to ensure the reliability of modeling and to know the dynamic action of modeled structures, the molecular simulation is performed through Gromacs MD package (http://www.gromacs.org) [39, 40]. The modeled structures are solvated in the TIP3P water model in the presence of OPLS-AA force field, the solvated structures are energy minimized using steepest descent method, terminating when maximum force is found smaller than 100 KJ mol-1 nm-1 [41, 42]. Water is used as a solvent when energy minimization is used to get rid of the poor atomic contact [43]. Total simulation was run in the NPT ensemble with a time step of 2fs, maintaining a temperature and pressure of 300 K and 1 bar, respectively [44, 45]. Minimized structures were equilibrated over a 10-ns timescale after 1ns of NVT. VMD (https://www.ks.uiuc.edu/Research/vmd/) is a molecular visualization program that is used to conduct the trajectory analysis [46].

Protein structure Analysis

The model structures from both the threading method and homology modeling method is validated using SAVES server, Ramachandran's map was drawn using PROCHECK [47]. The ProSA program was used to perform a comprehensive error check on the model protein structure and to compute the normalized Z-score for the model protein and its corresponding template molecules in order to establish whether or not the model structure is within the range of a high-quality experimental structure with a comparable shape and size [48]. The evaluated and error free structure was further chosen for the study.

Protein-Protein docking

Patch Dock, a geometry-based molecular docking technique (http://bioinfo3d.cs.tau.ac.il/PatchDock), is used to perform protein-protein docking in order to gain insight into the interactions between Glycam 1 and L-selectin [38, 49]. Allocating molecules into concave, convex, and flat patches, the patch dock method operates on the basis of molecular shape complementarity [50]. The shapes are matched with various complementarities and in order it generates different transformations [51]. Here, RMSD clustering was used to eliminate duplicate solutions based on the input of both proteins set to the default value of 4Å [49]. The output is refined using the firedock, which refine the protein-protein complex files for the purpose of understanding the clear interactions [52, 53]. PDBsum chain interactions are used to evaluate the top 20 postures and results. The chimera visualization tool is used to see how different surfaces interact with one another [54, 55].

Results and Discussion

Model Building

High resolution structure of Glycam1 and L-selectin proteins in *Bos Taurus* has not yet been determined experimentally (X-ray or NMR). Suitable homology modeling templates were located via a BLASTP search of the PDB using the default settings. If the similarity between the target and template is greater than 30%, then the suitable template can be selected and the sequences can be aligned. For L-selectin, we found PDB id 3CFW based on the maximum identity with high score and lower e-value, showing the similarity of 82% is used as templates for homology modelling. But for the Glycam1, there is lack of suitable template for modeling and this forced us to follow different procedure modeling these two proteins. In contrast with L-selectin homology modeling, the Glycam1 is modeled by using the threading method. L-selectin sequence alignment between target and template were done using ClustalW program, characterized by some insertions and deletions in the loop regions. Both the final model of L-selectin and Glycam1 is represented in figure 1a and figure 1b, which shows that the L-selectin is linear is shape and Glycam1 is packed. The homology modeled L-selectin shows 7 sheets, 7 beta hairpins, 1 psi loop, 5 beta bulges, 22 strands, 2 helices, 33 beta turns, 1 gamma turn, 11 disulphides as its protein framework (Figure represented in figure S1 and S3). While the Glycam1 shows the secondary structure of 11 helices, 21 helix-helix interacs, 32 beta turns, 2 gamma turns (Figure represented in figure S2 and S4). Both modeled proteins are fine, and we found, there is no missing loops are amino acids in the modeled structures followed by a rigorous refinement of the model by means of molecular dynamics and energy minimization.

Molecular Dynamics Simulations

L-selectin and modeled Glycam1 protein were shown to be relatively stable during explicit solvent MD simulations. The stability of the protein was obtained by the RMSD plot evaluated from VMD working panel simulation quality analysis. In Molecular Dynamics simulation, RMSD of the C-α of protein model for both the model proteins are analyzed to understand the stability in solvent dynamic environment. The RMSD assessments of C-α atoms from MD simulation are plotted in nm with the respect to time-dependent function 10ns (nanoseconds). From the beginning of simulation to the entire simulation, the RMSD plot shows strong support the model protein by showing constant RMSD deviation. While the model structure in MD simulation here is shown as an evidence for stable along with the intention of lively response in dynamic movement. The overall simulation process is a 10ns timescale and represent up and down deviations in RMSD plot in figure 2. Average indices show that the RMSD of Glycam-1 is 0.2216 nm and for L-selectin the RMSD is 0.4192 nm. While comparing both the proteins, L-selectin shows fluctuations in the 10ns simulations than the Glycam-1, and this is due to L-selectin is linear in shape. Glycam-1 shows compact in shape and it makes the simulations to deviates low with the average RMSD of Glycam-1 is 0.2216 nm. Overall, the RMSD of both L-selectin and Glycam-1 is showing stable without major fluctuations, and this makes to clear that our models are reliable to use for the molecular modeling calculations.

Structure Validation

The stable average conformation of the model proteins from the MD simulation is taken for the structural validation. Calculating the backbone and side chain conformations with Procheck and Errat from SAVES server ensured the quality and dependability of the modeled structure. The percentage of Φ–ψ angles in core Ramachandran region is placed more in the favorable regions for both L-selectin and Glycam-1, 99.4% and 96.5% respectively in the protein structure satisfied both phi and psi dihedral angle distribution. In terms of disallowed regions of model protein for both L-selectin and Glycam-1, 0.6 % and 3.5 % respectively, which shows that the protein models are well placed in the allowed region (Figure 3a and 3b). From the errat, the overall quality of the protein model is said to satisfactory, as both the protein models passed the values of more than 70. Here the overall quality factor for L-selectin is 89.224 and for Glycam-1, it is 81.379 (details provided as SI figure S5 and S6). Based on the Ramachandran plot and Errat analysis, it is clear to use the model for the molecular modeling calculations.

Protein-Protein Interactions

Protein-protein interactions (PPIs) are important mechanism in almost every cell function, and for that the proper understanding the interactions between those proteins are mandatory in normal and disease states. Biomolecular relationships or associations can be analysed through the protein-protein interactions, and here we have carried out the protein-protein interactions through in silico methods [12-14]. Combine algorithm of Patch dock and fire dock provides 10 best complexes that are having interactions with each other. We have analysed all the interactions and through the literature, the Glycam-1 is interacting with the L-selectin in the region of Lewis x (Lex), and by considering that, we got the top hit interactions by showing the protein-protein binding in exact region. We believed that, the top hit is the best by its scoring values and the experimental evidence of replacing the sLex (N-acetyl glucosamine and MAN) with template to the target matches the location, that specially section the allocation for protein-protein interactions. The protein-protein interactions are performed based on shape complementary and in final, it delivers the exact phenomenon of Glycam-1 binding over the sLex region. Computational modelling of the quaternary structure of complexes generated by two or more interacting biological macromolecules is known as "macromolecular docking." By using this method, we conform that, the model protein of Glycam-1 has the tendency to bind with the model structure of L-selectin.

Surface Interaction Analysis

The interactions are showing between the L-selectin and Glycam-1, and for the detail understanding, we examined the protein-protein conformations, by using the surface interaction analysis. The surface of the proteins is coloured in difference represented in figure 4 and analysed for interactions between the L-selectin with sLex and Glycam-1. In figure 4, the long straight structure protein L-selectin can say as head and tail, and in this head, portion is having the hold of sLex and Glycam-1 in this region. For the figure representation, the PPI complex

is shown in 360° coloured with L-selectin in thick red, Glycam-1 in Pink and sLex with turquoise. This shows that there is a good interaction seen in between both the proteins and we can also see that the sLex is playing a role of interaction bridge through this surface interaction analysis.

Interaction Analysis

Literature evidence shows that, L-selectin is considerably expressed in myeloid cells and naïve T cells and for that, the common ligand carbohydrate sialyl-Lewis x(sLex) is required and both these are bind together with high binding affinity. This high binding affinity is controlled by nature of the carbohydrate backbone scaffold which carries sLex. Generally, it was reported that L-selectin ligand binding is significantly retracted while the cells are treated with GalNAc which leads blocking of O-linked sugar synthesis. sLex selectin interaction and its Ca^{2+} independent, this binds to an alternative region of selectin domain. Significant percentage of the native ligand of L-selection is belongs to the family of adhesion molecules are mucin type ligand which composed of O-linked sulfate or carbohydrate side chain [45]. The interaction of L-selectin and sLex might override chemokine-dependent integrin activation and based on this information, it is mandatory to report the interactions of L-selectin with sLex and Ca^{2+}. sLex and Ca^{2+} interaction with L-selectin is analyzed through Ligplot for understanding the amino acid contributions in biological mechanism [46]. The interacting amino acid with sLex and Ca^{2+} are represented in the figure 5, showing that Lys106 is showing the main interactions and in terms of Ca^{2+} the amino acids Glu118, Asn120, Glu126, Asn143 and Asp144 are showing strong interactions. To specify the interaction between both proteins, it is also needed to view the contribution of amino acids that play a role of interactions (Figure 6a). For that, we examined the interacting amino acids in figure 6b labelled as L-selectin as A-chain and Glycam-1 as B-chain. In L-selectin, the Trp39, Arg58, Tyr61, Thr62, Asp63, Ile67, Gln68, Asn69, Gly102, Thr103, Thr157, Ala158, Lys161, Pro162, Gly167, Gln170, Cys171, Val172, Asn181, Asp183 and Leu184 are the amino acids having the potently to bind with other protein. In terms of Glycam-1, the interacting amino acids Asn21, Lys22, Thr27, His28, Leu29, Glu30, Ala31, Gln32, Pro76, Gln77, Pro81, Lys82, Leu83, Leu85, Ser86, Lys89, Glu135, Lys138 Tyr139 and Ser145 are actively involved in interaction with L-selectin.

Conclusion

In the era of protein-protein interaction-based structure functions, it is known to mediate the cell-cell recognition and adhesion events through the protein - carbohydrate interactions. Generally, it was reported that L-selectin ligand binding is significantly abrogated when the cells were treated with GalNAc which leads blocking of O-linked sugar synthesis. Slex selectin interaction and its Ca2+ independent, this binds to an alternative region of selectin domain. Through this study we have conform this experimental proof and L-selectin interaction with Glycam-1 is major protein mechanism involves in immunize to lactation process. This binding mechanism regulates the leukocyte rolling, tethering on vascular endothelial cells and promotes selectin shedding, and for that the information for selective amino acids to target is missing. Through this study, the protein structure for L-selectin with sLex and L-selectin with Glycam1 are visualized and

reported. As far as, the structure of Glycam1 has not yet studied till its structure level and this is the first study to report the Glycam1 structure. Interaction between these proteins is visualized and amino acid contributions are represented in the interactions and surface analysis. Molecular interaction studies inform that Lys106 is having the hold with sLex and in terms of Ca2+ Glu118, Asn120, Glu126, Asn143 and Asp144 are showing the interactions. From this study, we tried to elucidate major structural details of protein L-selectin and Glycam1, which are lacking still now. We believe that this molecular insightful information provided will be beneficial for the future research in this dairy industry.

Declaration of Interest

All the authors confidently declare that there is nothing to declare.

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Figure 1

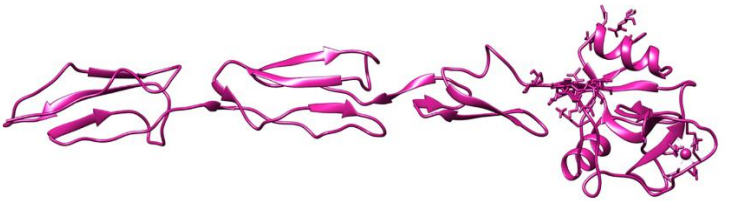

Figure 1a: 3D homology model structure of L-selectin from *Bos taurus*

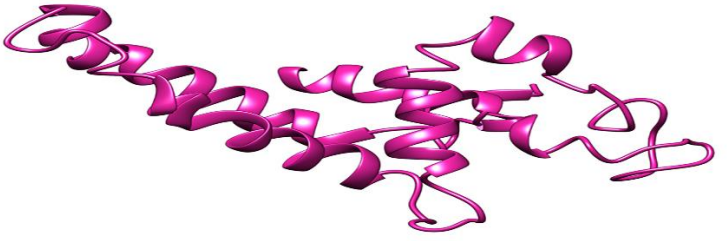

Figure 1b: 3D homology model structure of Glycam from *Bos taurus*

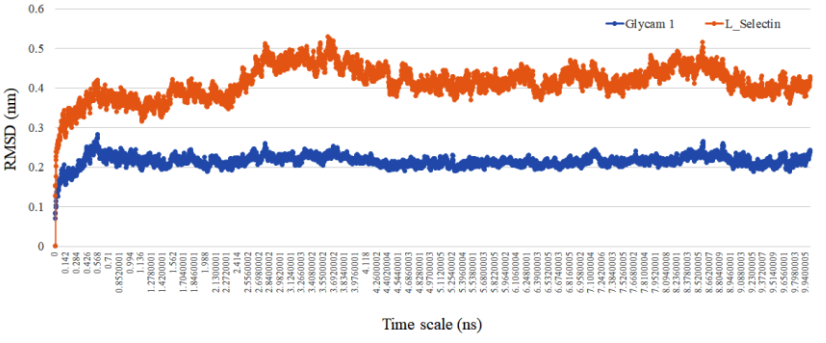

Figure 2: RMSD plot for the MD simulation of L-selectin and Glycam1 modeled proteins from *Bos taurus*

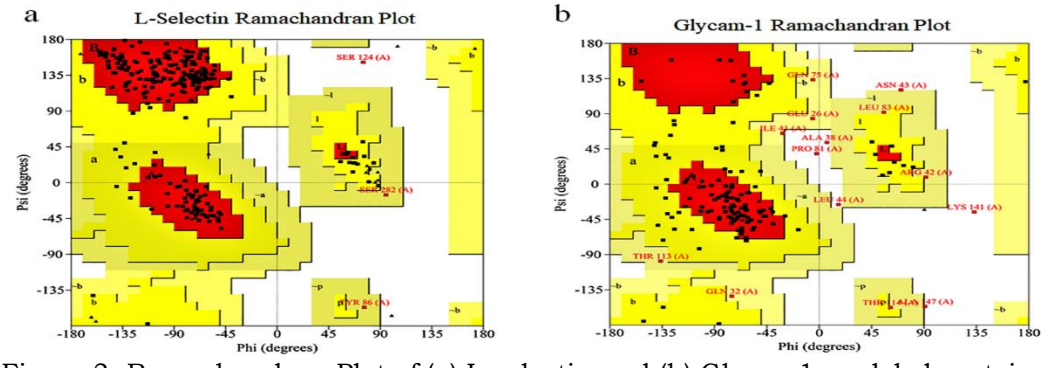

Figure 3: Ramachandran Plot of (a) L-selectin and (b) Glycam1 modeled proteins from *Bos taurus*

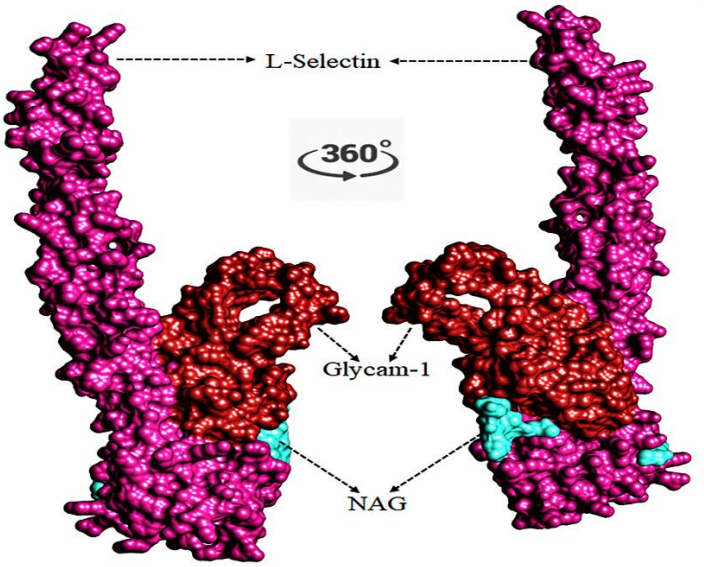

Figure 4: Surface interaction of Protein-protein interaction of L-selectin with Nag and Glycam1 showing the protein-protein binding in the NAG region

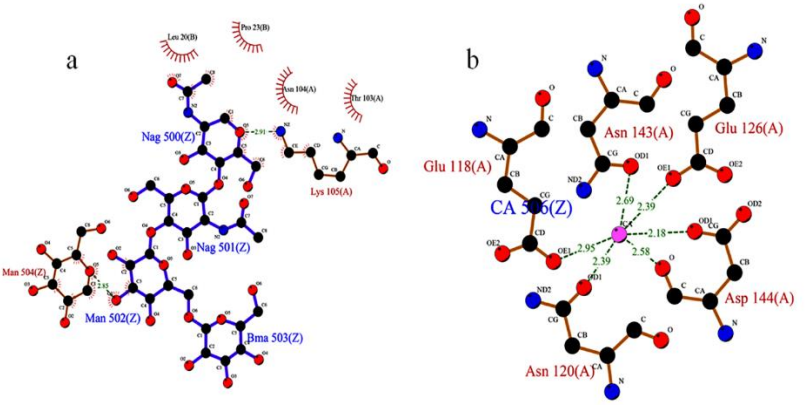

Figure 5: Molecular interaction of (a) co-factor and (b) metal atom interactions with L-selectin

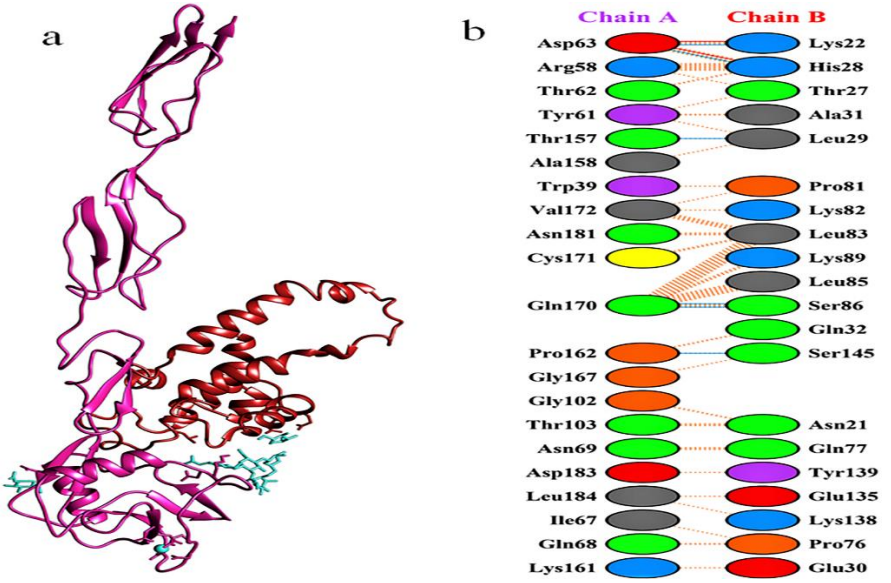

Figure 6: (a) Protein-protein interaction of L-selectin with Nag and Glycam1 showing the protein-protein binding in the NAG region (b) Residue wise participation of L-selectin (A-chain) and Glycam1(B-chain)

Supplementary Information

Mechanistic Insights of Glycosylation-dependent cell adhesion molecule-1 with L-selectin expressed in the lactating bovine mammary gland

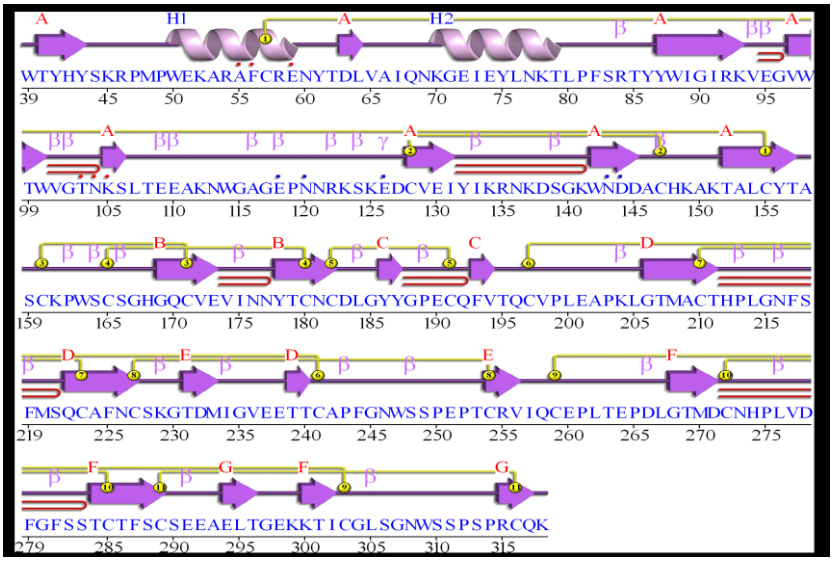

Figure S1: Secondary structure alignment of L-selectin homology model pattern from *Bos Taurus*

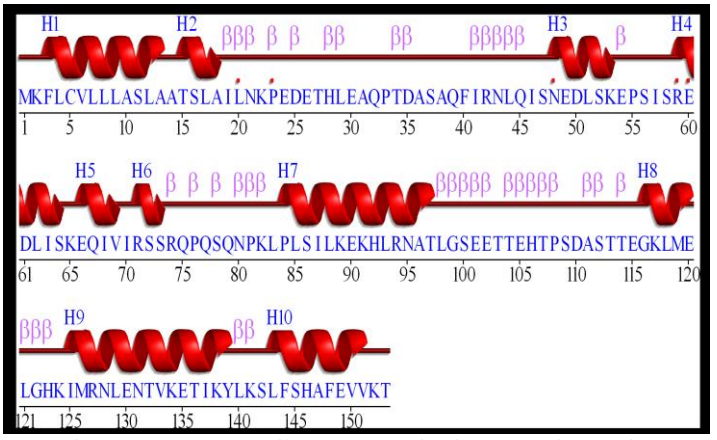

Figure S2: Secondary structure alignment of Glycam1 homology model pattern from *Bos Taurus*

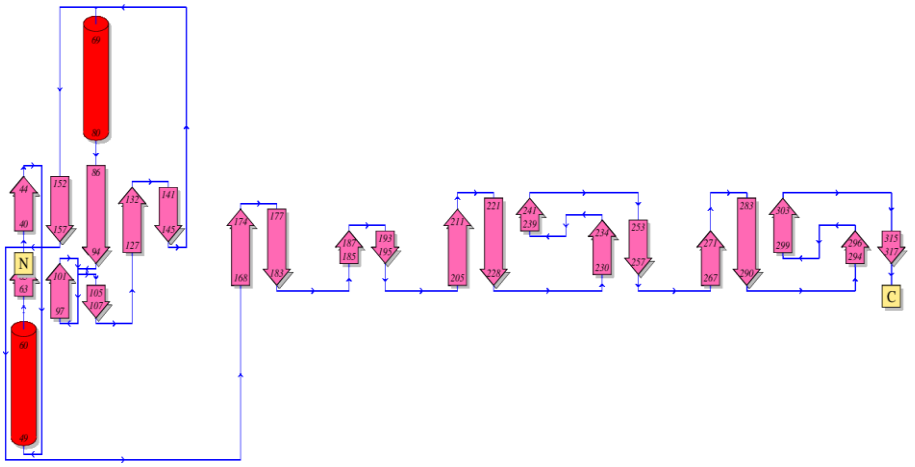

Figure S3: Network topology of L-selectin homology modeled protein

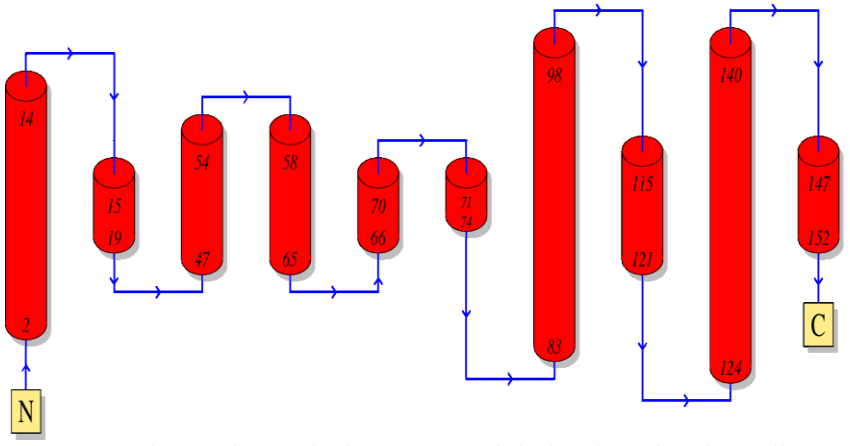

Figure S4: Network topology of Glycam1 modeled using the threading Method.

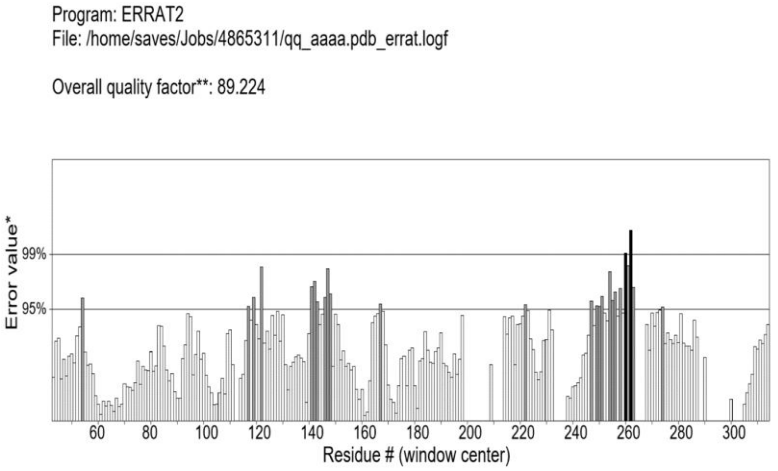

Figure S5: Quality of model protein L-selectin through Errat2 prediction

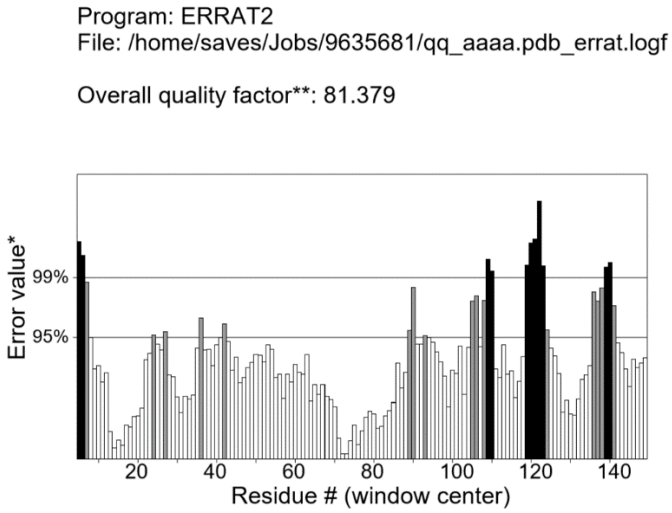

Figure S6: Quality of model protein Glycam1 through Errat2 prediction