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A Computational Investigation on Immunogenicity of Uricase from *Aspergillus Flavus* and *Candida Utilis*

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Uricase is a therapeutic enzyme that has applications in adjuvant chemotherapy and the management of treatment-resistance hyperuricemia. It is a key element in gout treatment, a type of arthritis affecting humans due to increased serum uric acid levels. Two available formulations of uricase from *Bacillus fastidious* and *Arthrobacter globiformis* were characterized by their high immunogenicity, resulting in the drug's inactivation and hypersensitivity reactions in many patients. This research focuses on protein engineering to find a substitute to PEGylated enzymes with less immunogenicity as an intrinsic characteristic of the protein. We used *in silico* techniques to spot and modify epitope areas of uricase from *Aspergillus flavus* (*Af*) and *Candida utilis* (*Cu*), and to decrease the immunogenicity. Both Uricase B-cell epitopes were predicted using surface accessibility and hydrophilicity. Mutations were made to the hot-spot residues to diminish the epitope's antigenicity. Also, molecular docking was used to examine the effect of mutation on uricase activity and stability. Immunoinformatic analysis was done to clarify the structural aspects of the immunogenicity of uricase. For this purpose, the prognostication of immunogenic and allergenic epitopes in uricase structure was performed by using immunogenic peptides relative frequency. To the best of our knowledge, this is the first report of an *in silico* investigation aimed at decreasing the immunogenicity of uricase from *Aspergillus flavus* and *Candida utilis*.

Keywords: Uricase, Gout, *In silico* mutagenesis, Hypersensitivity, Immunogenicity

INTRODUCTION

Uricase (E.C.1.7.3.3) is a promising therapeutic enzyme that comes under the category of oxidoreductase. Uricase has been isolated from different species such as bacteria, yeast, and fungal sources. Depending on the source, the structure of uricase varies. Tetrameric uricase (consisting of four subunits) from *Aspergillus niger* is barrel-shaped, whereas uricase isolated from *Bacillus fastidious* has a tunnel shape (Fig. 1). Each subunit consists of two domains [1-3]. Usually, the molecular weight of uricase ranges from 145-150 kDa. A circular dichroism study was performed, and it was found that each domain consists of four strands of antiparallel β strands and two antiparallel α strands [4].

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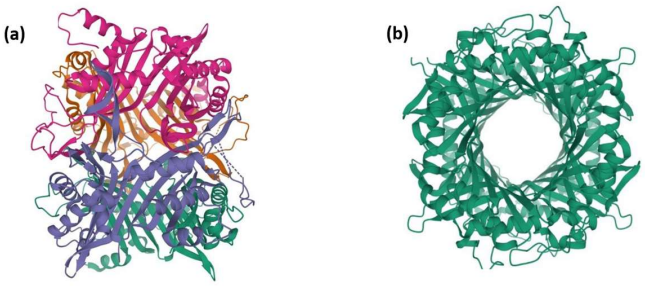

Fig. 1. 3D structures of a) *Bacillus fastidious* and b) *Aspergillus flavus* uricase.

Uricase is absent in humans and apes because of primate evolution due to mutation in exon 3 [5,6]. Uric acid has low solubility, and it cannot excrete through the kidney. Because of this, in some humans, the uric acid gets accumulated in the form of crystals in the liver, muscle, and

joints, leading to gout and hyperuricemia. In developed countries, 1-3% of adults are affected by gout hyperuricemia. The main role of uricase is to accelerate the oxidative breakdown of uric acid to form allantoin. Allantoin has higher solubility than uric acid resulting in effortless excretion. This makes uricase a potential drug for treating hyperuricemia over the other chemical drugs such as allopurinol and febuxostat. These chemical drugs cause adverse effects, which sometimes lead to lethal [7-11].

In 1957, London and Hudson reported intravenous uricase treatment in two persons, one with chronic gout. This medication resulted in a quick decline in serum uric acid levels and an increase in allantoin renal excretion. For the prevention and treatment of hyperuricaemia caused by chemotherapy in patients with haematologic malignancies, the European Medicines Agency (EMA) authorised rasburicase (Fasturtec®) in February 2001. Patients with persistent gout, who are intolerant or unresponsive to traditional therapy, can now take pegloticase (Krystexxa®), a recombinant polyethylene glycolated recombinant uricase (PEGylated). Clinical evaluation of uricase from *Candida utilis* (pegloticase) is currently in progress [12-14]. Uricase has been utilised not just for therapeutic purposes, but also for diagnostic purposes. The urine or serum uric acid concentration has been quantified using uricase-based biosensors [15,16].

Despite the fact that uricase has several advantages and is utilised in various applications, it also has some drawbacks. Uricase induces hypersensitivity and allergic reactions [17]. Rasburicase produces hemolytic anemia owing to glucose-6-phosphate dehydrogenase deficiency. It affects nausea, vomiting, and diarrhea. Patients taking rasburicase regularly reported decreased blood oxygen levels, breathing difficulties, and skin rashes. Pregnant women are not allowed to take this dosage due to these side effects [17]. Another FDA-approved uricase, Pegloticase, was associated with mild to moderate adverse effects in 77% of patients. Infusion responses and gout flares were common side effects of pegloticase, which led to some patients stopping treatment [18]. When the serum uric acid level exceeds 6 mg ml^{-1}, pegloticase is no longer administered. Additionally, it contributes to dyslipidemia and metabolic diseases [18].

These kinds of adverse reactions are due to immunogenicity and allergenicity of uricase [19,20]. PEGylation is a common approach for reducing the immunogenicity of the enzyme by covering its allergenic epitope. But it is biochemical method, which also causes an adverse effects such as decreasing the catalytic efficiency of an enzyme and also triggers immune response like antibody secretion, which is more specific to PEGylated enzyme [21]. In order to overcome the disadvantages, a molecular biological approach called site directed mutagenesis should be taken. Site directed mutagenesis is a cost effective and time consuming technique, which may sometimes has a diversion from our intentional site of mutation [22]. Therefore, the present study deals with structural modeling of uricase, immunogenicity, allergenicity analysis of uricase from *Aspergillus flavus* and *Candida utilis* (because it clinically approved that uricase was isolated from *Aspergillus flavus* and *Candida utilis*) and *in silico* mutagenesis for reducing the immunogenicity. The study focuses on protein engineering to find a substitute to PEGylated enzyme, with less immunogenicity that is an inherent character of protein. To this end, in silico methodologies were used to overcome the bottlenecks that arise during the development of therapies. This study uses computational methods to identify enzyme B cell epitopes, modify the immunogenicity of a protein by creating point mutations of its epitopes, perform molecular modelling for enzyme model generation, and to do *in silico* docking techniques with Autodock vina to evaluate the substrate's binding affinity with uricase variants. Also, validating the mutated model for stability by I-Mutant server was performed.

MATERIALS AND METHODS

Sequence Retrieval

Structure of uricase from *Aspergillus flavus* (1R56) was retrieved from PDB (Protein Data Bank). The structure of uricase from *Candida utilis* has not been predicted yet. So, protein modelling was done by using various computational tools or online servers.

Protein Modelling

Uricase protein sequence was retrieved from UNI- PROT and structure of uricase from *Candida utilis* has been modelled by several protein modelling tools

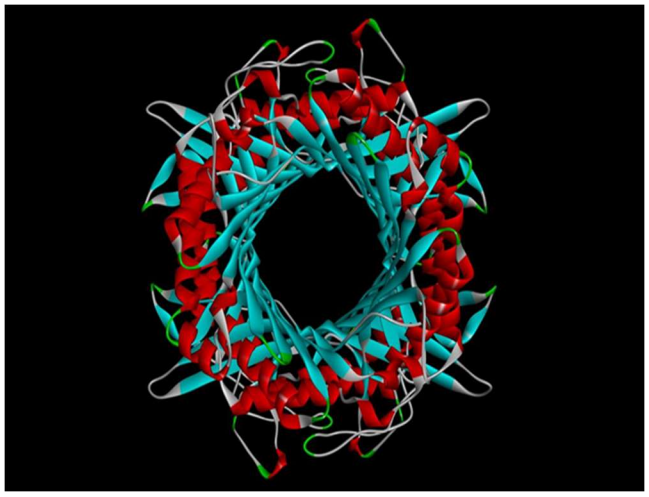

Fig. 2. Modelled structure of uricase from *Candida utilis*.

such as SWISS-PROT server, I-TASSER server and Modeller 9.21 [23-25]. Protein BLAST has done for selecting the best template sequences. For modeller, four template sequences were selected based on the highest sequence identity with our unknown sequence. By comparing the predicted structure of uricase, among the above mentioned tools, SWISS-PROT gave the best modelled structure of uricase shown in Fig. 2. Several structural parameters such as Pro A web score, verify 3D, and Ramachandran plot for uricase structure obtained from three different modelling tools analysed by SAVES server (https://saves.mbi.ucla.edu/).

B Cell Epitope Prediction

B-cell epitope identification is the fundamental step to study the immunogenicity of the protein. Analysis of B-cell epitope was performed by either on structural basis or by sequence data alone [20,26,27]. B-cell epitope was predicted to *Candida utilis* and *Aspergillus flavus* by using IEDB tool. B- cell tools (BepiPred2.0) by Emini surface accessibility and surface hydrophilicity [20,27,28] prediction methods were used in the IEDB database.

In Silico Mutagenesis

In silico mutagenesis is a method in which the amino acid residues of protein are changed (mutated) in order to improve the characteristics of protein by decreasing the immunogenicity [29]. To decrease the immunogenicity of the enzyme, *in silico* mutagenesis was performed using pymol [29,30]. In uricase from *Aspergillus flavus*, amino acid residues Ser124 and Glu166 were replaced by Ile, Glu22, Glu125, Glu126 and Asp223 were replaced by Phe. In uricase from *Candida utilis*, Glu 299 was replaced by Val, Lys28 was replaced by Ile, Asp53 and Pro27 were replaced by Trp. The stability of mutated protein was examined using I-Mutant 2.0 (http://folding.biofold.org/i-mutant/i-mutant2.0.html). Changes were allowed if the difference in Gibbs free energy was positive, which meant that the stability of the protein was getting better. difference in Gibbs free energy was calculated using the formula of ΔG(mutein) – ΔG(wild type protein) [31].

Molecular Docking

Molecular docking techniques predict ligand binding affinities. All docking studies used default parameters for accurate results. AutoDock Tools was used to prepare protein and ligand pdbqt files and create grid boxes using AutoDock Tools (ADT). ADT gave the protein polar hydrogens, Kollman charges, solvation parameters, and fragment volumes. AutoDock stored the PDBQT file. Using AutoGrid, a grid map was created. AutoDock Vina was used to dock the ligand, using information in the configuration file about the protein, and the grid box properties. AutoDock Vina uses iterated local search global optimizer. The pose with the lowest binding energy or affinity was taken and matched up with the structure of the receptor for further study [32,33].

Both native and mutated uricase from *Candida utilis* and *Aspergillus flavus* were docked to their substrate (uric acid) to examine the binding affinity of the enzyme after mutation. Autodock Vina was used to perform molecular docking [34]. The Autodock Vina input files were built using AutoDock Tools 1.5.6. AutoDock required polar hydrogen atoms and gasteiger charges to prepare files. The more restrictions were left unspecified for Autodock Vina. The ligand binding affinity is expressed as a negative score in Kcal mol^{-1}. The performance of various docking software was analyzed and it has been observed that Autodock Vina ranks the top most among the other docking softwares [35-37].

RESULTS AND DISCUSSION

Protein Modelling

The three-dimensional (3D) structure of a protein provides crucial insight into its function, which is essential for the development of effective experimental designs. Homology modelled protein or enzyme structures are useful for organising and assessing biological experiments, when no experimental structures are available for proteins or enzymes of interest [38]. Structure of uricase from *Candida utilis* was modelled using several modelling tools like Swiss model, Modeller and I-Tasser. The modelled structure was then examined by SAVES server, and verify 3D scores and Ramachandran plot values are listed in Table 1. Model quality was assessed by the SWISS-MODEL server using both the QMEAN4 global score and the QMEAN Z-score. The SWISS-MODEL workspace's QMEAN4 score was used to the generated target model to determine its overall quality. Overall model quality was represented by the ProS A web z-score. As a numerical value, it is plotted alongside the z-scores of all experimentally determined protein chains in the current PDB. Verify 3D checks if an atomic model (3D) is compatible with its own amino acid sequence (1D) by giving each atom a structural class based on where it is, what is around it (alpha, beta, loop, polar, nonpolar, *etc*.), and comparing the results to good structures. Verify 3D showed that at least 80% of the amino acids had a 3D/1D score of >= 0.2. Ramachandran plot showed that the best model would be expected to have more than 90% amino acid residues in the most favoured regions. From Table 1, it has been observed that Swiss model-2 has Pros A web Z-score -7.45, Verify 3D score -83.31%. These are values obtained from Ramachandran plot: Favoured region -96.6%; allowed region -3.1% and outlier region -0.3%.

Surface Accessibility

Surface accessibility was prognosticated using IEDB tools (http://tools.iedb.org/mhcii/) for uricase from *Aspergillus flavus* and *Candida utilis* [39]. It has been noted that the main contributors to the creation of antigenic sites or epitopes in an enzyme are either uninterrupted sequential areas or different antigenic determinant groups [22]. Antigenic characteristics such as Emini surface accessibility, Karplus and Schulz flexibility, and Parker hydrophilicity were used to anticipate continuous B-cell epitopes for the therapeutic enzyme uricase, with the goal of decreasing its clinical immune-reactiveness. According to a report using a propensity scale for each of the 20 amino acids, most antigenic areas of proteins have more hydrophilic and charged residues when compared to hydrophobic residues [40-42]. Table 2 shows the anticipated B-cell epitope peptides for uricase from *Aspergillus flavus* (*Af*) and *Candida utilis* (*Cu*) based on their Emini surface accessibility, Karplus and Schulz Flexibility and Parker hydrophilicity scores. The yellow regions are predicted epitopes of the enzyme uricase. It has been observed that amino acids such as serine (124S), glutamic acid (166E) in *Aspergillus flavus* (Fig. 3A) and glutamic acid (299E), and Lysine (28K) in

Table 1. Structural Analysis of Uricase from *Candida Utilis*

S.No	Modelling toolused	Pros A webZ-score	Verify 3D	Ramachandran plot		
				Favored region	Allowed region	Outlier region
1	SWISS model 1	-7.36	81.48%	96.3%	3.3%	0.3%
2	SWISS model 2	-7.45	83.31%	96.6%	3.1%	0.3%
3	Modeller model 1	-7.03	77.89%	96%	2.7%	1.3%
4	Modeller model 2	-7.6	79.89%	95.7%	3.3%	1%
5	Modeller model 3	-7.14	79.87%	96.3%	2.7%	1%
6	Modeller model 4	-6.96	82.18%	95.7%	2.7%	1.7%
7	Modeller model 5	-7.34	84.16%	94.7%	3.3%	2%
8	I-Tasser	-7.75	83.83%	87.4%	8%	4.7%

Table 2. B-Cell Epitopic Scores of Uricase from *Aspergillus Flavus* and *Candida Utilis*

Strain	Method	Amino acid	Score
Aspergillus flavus	Surface accessibility	124S, 166E	5.296, 3.724
		124I, 166I (After mutation)	2.928, 1.593
	Hydrophilicity	22E, 125E, 126E, 223N	6.029, 6.6, 7.0
		22F, 125F, 126F, 223F	3.6, 1.793, 2.143
		(After mutation)	
Candida utilis	Surface accessibility	299E, 28K	5.821, 7.641
		299V, 28I (After mutation)	2.234, 3.586
	Hydrophilicity	53D, 27P	6.443, 4.943
		53W, 27W (After mutation)	3.214, 3.586

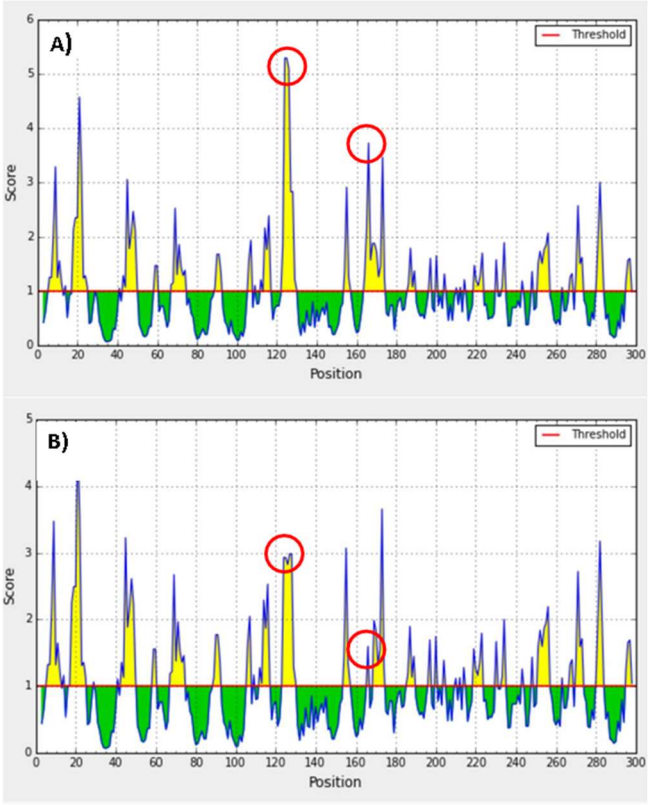

Fig. 3. A) Surface accessibility score of *Aspergillus flavus* before mutation. B) Surface accessibility score *Aspergillus flavus* after mutation.

Candida utilis (Fig. 4A) are responsible for the increasing immunogenicity of uricase. Therefore, in order to decrease the immunogenicity of uricase, these amino acids are replaced by serine, glutamate, isoleucine, asparagine, phenylalanine, lysine, valine, aspartate, proline, tryptophan; the stability of enzyme was verified by I-mutant server. It has been found that isoleucine (124I, 166I) in *Aspergillus flavus* (Fig. 3B) and valine (299K), and isoleucine (28I) in *Candida utilis* (Fig. 4B) exhibited the higher stability of uricase. Increased immunogenicity is correlated with increasing hydrophilicity at the protein's epitopic location. Thus, Bander's attempt for a Monoclonal Antibody (^{177}Lutetium-Labeled J591) to Prostate-Specific Membrane Antigen, reduced antigenic scores by replacing one of the epitopic site residues with hydrophobic valine and leucine [43].

Hydrophilicity

Similar to surface accessibility, hydrophilicity of enzyme was also ascertained by IEDB tools (http://tools.iedb.org/mhcii/) [44]. The immunogenicity increasing amino acids from both strain (*Aspergillus flavus and Candida utilis)* of the enzyme is glutamic acid (22E, 125E, 126E), asparagine (223N) in *Aspergillus flavus* (Fig. 5A) and aspartic acid (53D), and proline (27P) in *Candida utilis* (Fig. 6A). Their amino acids were replaced by several amino acids and its stability after mutation was verified using I-mutant server. It has been observed that phenylalanine (22F, 125F, 126F, 223F) in *Aspergillus flavus* (Fig. 5B) and tryptophan (53W, 27W) in *Candida utilis* (Fig. 6B) exhibited the higher stability of uricase.

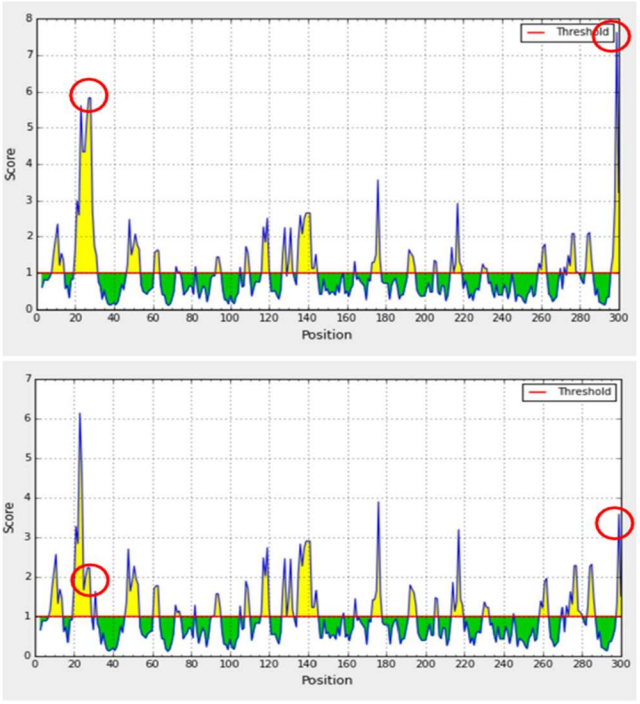

Fig. 4. A) Surface accessibility score of *Candida utilis* before mutation. B) Surface accessibility score *Candida utilis* after mutation.

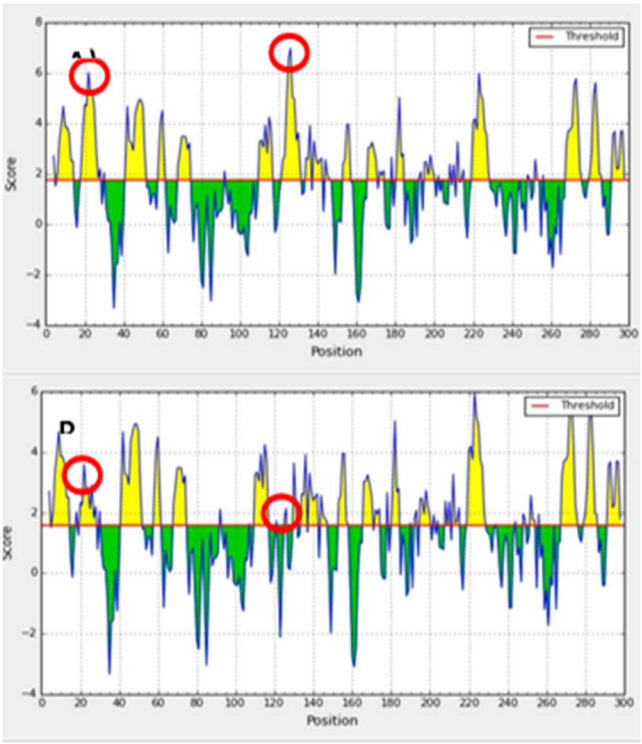

Fig. 5. A) Hydrophilicity score of *Aspergillus flavus* before mutation. B) Hydrophilicity score of *Aspergillus flavus* after mutation.

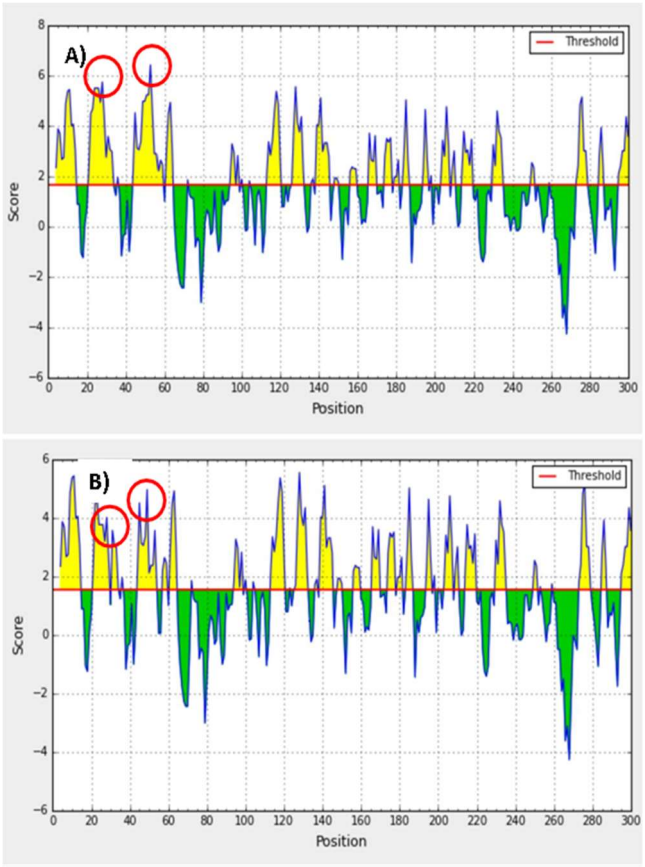

Fig. 6. A) Hydrophilicity score of *Candida utilis* before mutation. B) Hydrophilicity score of *Candida utilis* after mutation.

Molecular Docking

Initially native uricase from *Aspergillus flavus* and *Candida utilis* was docked with its substrate (uric acid) using Autodock vina and their binding affinity are listed in Table 3. *In silico* mutagenesis has done in *Aspergillus flavus* and *Candida utilis* using Pymol. After mutation, uricase from *Aspergillus flavus* and *Candida utilis* was successfully docked with uric acid to examine the binding affinity of the enzyme with native uricase binding affinity (Table 3). From the above table, it has been observed that a promising binding affinity was obtained after *in silico* mutagenesis, indicating that catalytic efficiency of the enzyme was not decreased to a great extent after mutation. The interactions between uricase from Aspergillus flavus and Candida utilis with their substrate uric acid are s hown in Fig. 7.

Table 3. Binding Affinity of Native and Mutated Uricase

Strain	Model	Binding affinity
Candida utilis	1	-6.3
	2	-5.7
	3	-5.5
	4	-5.5
	5	-5.4
Aspergillus flavus	1	-5.5
	2	-5.4
	3	-5.3
	4	-5.1
	5	-5.0
Mutated *Aspergillus flavus*	1	-5.4
	2	-5.4
	3	-5.3
	4	-5.3
	5	-5.3
Mutated *Candida utilis*	1	-5.7
	2	-5.7
	3	-5.5
	4	-5.5
	5	-5.5

Recent developments in protein engineering enable researchers to minimize immunogenicity, enhance protein stability, and improve function [40,45,46]. Creating mutations may help improve the quality of enzymes or protein drugs. A conceptual approach to providing a reference for experimental purposes has been previously published, using the modification of an antigenic portion of horseradish peroxidase [26]. Researchers used *in silico* technologies to detect B-cell epitopes and then, they selected the suitable amino acid candidates depending on the *in silico* data. Using computational tools, these strategies can be used to create vaccines [26,47]. Various therapeutic enzymes were studied for reducing the immunogenicity by computational tools reported in Table 4.

CONCLUSIONS

The FASTA sequences of uricase from *Aspergillus flavus* and *candida utilis* were retrieved. The structure of uricase (from *Aspergillus flavus*) was retrieved from Protein Data Bank (PDB) and for uricase (from *candida utilis*), the structure was modelled by SWISS PROT and Modeller. After verifying the parameters such as ProA Web score, verify 3D score and Ramachandran plot in SAVES server, the structure modelled by SWISS PROT was taken for further analysis. The epitopes which are responsible for

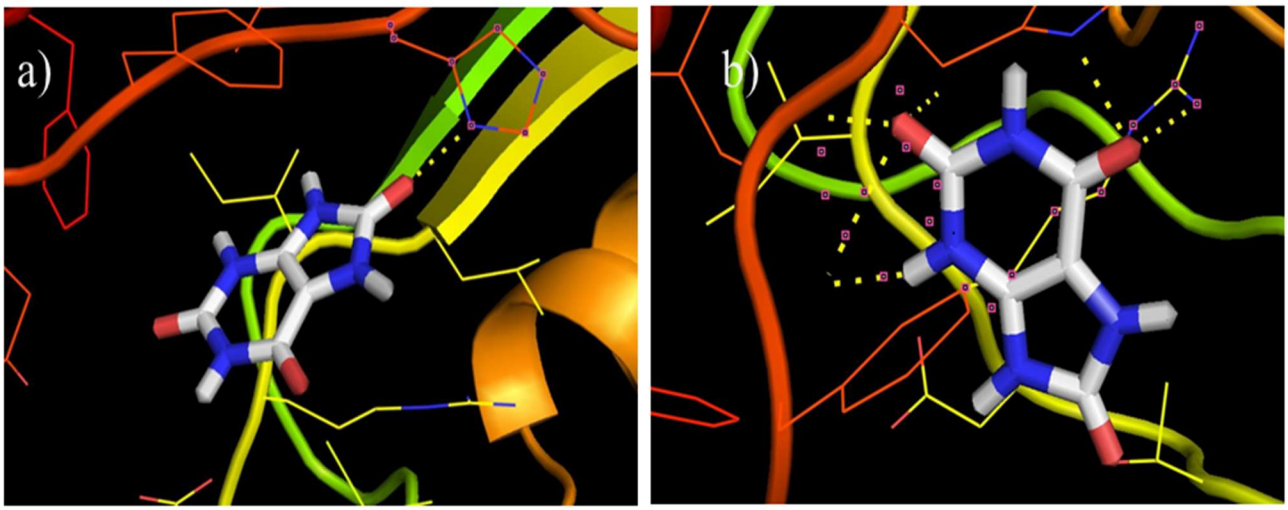

Fig. 7. a) Interaction between uricase from *Aspergillus flavus* with uric acid. Dotted yellow lines represents hydrogen bond. b) Interaction between uricase from *Candida utilis* with uric acid.

Table 4. Immunogenicity Reduction Study by Different Enzymes Using *In Silico* Tools

Enzyme	Source	Mutations	Inference	Ref.
L-Asparaginase	Erwinia chrysanthmi	H240A, Q239A D235A K265A, E268A.	Therapeutic protein immunogenicity was reduced by using point mutations.	[48]
L-Asparaginase	Escherichia coli	K314C, D78L, Q307P,	The immunogenicity has decreased as a result of replacing an epitopic site residue with a hydrophobic one.	[22]
	Pectobacterium carotovorum	E231V, D145L, D229G		
Arginine deiminase	Mycoplasma hominis	E304L	Proteins C and N terminal moieties are largely responsible for the majority of epitopic conformers	[40]
Botulinum Toxin Type A	Bacteria	E510F, T1062F, N1089M, E510F, T1062F, E1080W, N1089M	The protein with reduced immunogenicity was created by substituting nonpolar amino acids for the immunogenic residue.	[49]
Uricase	Arthrobacter globiformis	T159W, D169C, N264W and Y203D	The greatest strategy to lessen immunogenicity was to replace polar residues with hydrophobic ones	[20]
	Bacillus fastidious	S139 V, K215W, G216 F and I172 P		
Uricase	Aspergillus flavus	S124I, E166I, E22F, E125F, E126F, N223F	Most of the antigenic regions of the enzyme were reported to have more hydrophilic and charged residues when compared to hydrophobic residues	This study
	Candida utilis	E299V, K28I, D53W, P27W		

higher immunogenicity of enzyme was predicted by IEDB tools and listed. The active site residues of uricase from *Aspergillus flavus* was acquired from Protein Data Bank and the active site amino acids that contribute to higher immunogenicity were mutated and docked. Similarly, blind docking was performed for the modelled structure of uricase from *Candida utilis,* as the active sites were unknown. In both cases, binding affinity of docked mutated uricase was compared with the docked native uricase. From the results of this study, it has been found that the mutated amino acids were prominently contribute a major part in decreasing immunogenicity, despite a slight decrease in binding affinity. This work can be future reference for wet lab experiments regarding development of efficient uricase for therapeutic uses.

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