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An *In-Silico* Study of Stable and Environment-Friendly *Oryza sativa* Urease

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Abstract: Urea is one of the most extensively used fertilizers in agriculture but has a detrimental impact on the environment. One of the strategies to reduce this impact can be engineering modified plants containing urease enzyme with a considerably higher affinity for urea so that the urea applied in the fields can be significantly reduced. In this study, we have selected *Oryza sativa* Urease and generated stable mutants having a high affinity for urea. We modeled the 3D structure of the enzyme and identified the potential binding sites by analyzing the binding sites of similar proteins, i.e., 48 urea binding proteins. We found that mutation of Arg578 with Cys near the substrate-binding site of *Oryza sativa* Urease leads to a stable mutant protein that has a higher binding affinity for urea. This study will lead to a generation of environment-friendly, stable, genetically modified rice crop that consumes lesser urea, without compromising with crop productivity.

Keywords: *Oryza sativa* Urease; molecular docking; high urea affinity; molecular modelling; mutation analysis.

1. Introduction

Plants suffice their nutrient requirements due to the presence of Nickel-dependent metalloenzyme- Urease (EC 3.5.1.5), present in various plant species as the housekeeper enzyme, playing a vital role in catalyzing the hydrolysis of urea, converting it to ammonia in the cytosol, which further acts as a substrate for Nitrogen assimilation in plants [1]. With an estimated production of 480.13 million metric tons in 2016-17, indicated by USDA (United States Department of Agriculture), *Oryza sativa* (Rice) is one of the predominantly grown cereal crops worldwide, crucially depending on urea as the main source of nitrogen fertilizer [2], which is accessible to plants, only after its hydrolysis, mainly by microbial urease, followed by plant ureases [3, 4].

Widespread application of urea for paddy growth has a detrimental impact on the ecosystem, due to the high activity of microbial ureases in the soil leading to ammonia volatilization, phytotoxicity, Nitrate accumulation, suspended seed germination [5], leaching, contamination of nearby water bodies, soil acidification, etc. [6,7]. Similar harmful effects of excess of another nutrient- Phosphorous, have been studied, and novel methods have been

designed to reduce its impact on the environment [8]. The different strategies employed, such as developing hydrophilic polymers for controlled ammonia release [9], experimenting with ground cover rice production system [10], use of membrane encapsulated starch-g-PLLA urea fertilizer [11], designing urease inhibitors [12] have not been completely successful**.** The reduced soil urease activity should be accompanied by increased plant urease activity**.** However, not much has been investigated on targeting the ureolytic activity of *Oryza sativa* urease itself, which could potentially enhance the urea metabolism and uptake by plants, preventing loses due to ammonia volatilization, ultimately reducing the urea dependence quantitatively, thus, limiting its adverse effects [3, 13-20].

In our study, we designed an environment friendly and stable *Oryza sativa* Urease mutant so as to enhance the enzyme affinity for urea. This can be used to generate genetically modified rice species containing urease that has a high affinity for urea, such that the amount of urea applied in the fields can be significantly reduced. Thus, large scale production of rice crops will not be accompanied by large scale urea applications in the fields, thus, saving the environment from its catastrophic effects.

2. Materials and Methods

2.1. Modeling of protein structure.

The amino acid sequence of Oryza sativa Urease (Accession ID BAB78715.1), retrieved from the NCBI database, was used to generate a full-length 3D protein structure with the help of I-TASSER server.

2.2. Binding-site analysis.

Multiple sequence alignment of 48 urea-binding proteins (retrieved from RCSB-Research Collaboratory for Structural Bioinformatics database) and *Oryza sativa* urease was done using Clustal Omega [21]. LIGPLOT analysis of urea-binding sites of closely related protein structures was done with the help of PDBsum [22]. Closely related proteins were structurally aligned and further superimposed with *Oryza sativa* Urease using UCSF Chimera to identify the binding pocket.

2.3. Generation of mutants.

UCSF Chimera [23] was used to visualize and select amino acid residues having a cutoff distance of 3.00 Å. We then obtained stabilizing amino acids on the mutation sites by CUPSAT server [24]. Chimera [23] was used to select the most probable rotamers of stabilizing amino acids as mutants, and clashes or contacts were removed so as to stabilize any strain remaining after mutation.

2.4. Docking.

The 3D structure of urea was obtained from the Chemspider database. Molecular docking of Urea and Oryza sativa urease and its mutants were carried out using the AutoDock software package (version 4.2) [25] as implemented through the graphical user interface AutoDock Tools (ADT 1.5.6). In docking grid box size of 40x40x40 points covering the whole protein structure was built. A grid spacing of 0.375 Å (approximately one-fourth of the length of the carbon-carbon covalent bond) and distances-dependent functions of the dielectric

constants was used for the calculation of the energetic map. Confirmation runs were generated by using Lamarckian genetic algorithm searches. Default settings were used with an initial population of 50 randomly placed individuals, a maximum number of 2.5×10^6energy evaluations, and a maximum number of 2.7×10^4generations. A mutation size of 0.02 and a crossover rate of 0.8 were chosen. The resultant complex structures were then selected based on binding free energy values.

3. Results and Discussion

3.1. Homology modeling.

The 3-D structure of *Oryza sativa* Urease was not available in the Protein Databank, so we modeled the structure using its amino acid sequence, which was retrieved from the NCBI database (Accession ID BAB78715.1). The enzyme consists of a single chain of 848 amino acid residues. I-TASSER server provided 5 full-length models of the protein, out of which the structure with a C-score (confidence score) of 2 (predicting the high quality of model) was selected (Figure 1). It consists of 34 α-helices, 11 of them being extremely short (with 3-4 amino-acid residues). Most of the helices are amphiphilic, interacting with the solvent, while a few are surrounded by loops. The 29 β-strands arrange themselves in 6 β-sheets, 2 buried in the core, while 4 are amphiphilic. The 2 β-turns consist of loops facing the solvent, while 8 β-strands form a barrel, making a hydrophobic pocket.

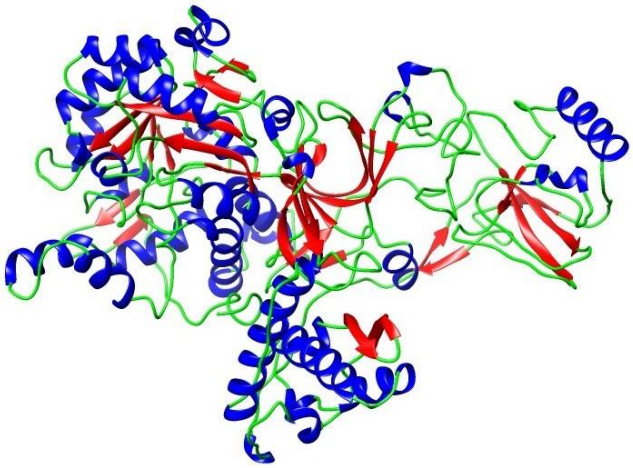

Figure 1. Structure of *Oryza sativa* Urease modeled by I-TASSER server. Blue color indicates helices, Red-beta-strands, Green-loops.

3.2. Binding-site analysis.

In order to perform the binding site analysis, we have first retrieved all the existing Urea binding proteins from the RCSB database. In the RCSB database, we have found 48 such proteins. Multiple sequence alignment *of Oryza sativa* Urease with the existing 48 urea-binding proteins was carried out. We found that *Oryza sativa* Urease has high similarity with 4 of the 48 existing urea-binding proteins. Among the 4 proteins, we have chosen Ricin-A from *Ricinus communis* (PDB id: 2R2X), Moesin from *Spodoptera frugiperda* (PDB id: 2I1J), Moesin from *Spodoptera frugiperda* (PDB id: 2I1K) for binding site analysis. We have not considered HLA-B*1501 from *Homo sapiens* (PDB id: 1XR9) for further binding site analysis because of the

absence of a urea-binding site in the chain C of 1XR9 (which has high similarity with *Oryza sativa* Urease). We studied the molecular interactions in the Urea-binding sites of the above mentioned 3 proteins with LIGPLOT analysis (Figure S1). From Figure S1, we can see that the residues Asn6 and Glu73 present in chain A of Moesin (2I1J), as well as 2I1K, were involved in forming hydrogen bonds with urea in its binding pocket, while the residues Met5, Asn74, Pro75, and Leu76 create a hydrophobic atmosphere around the pocket (Figure S1 a, b) [26]. The urea binding pocket in chain A of Ricin(2R2X) (Supplementary Figure S1 (c)) contains Gly121 and Val81, which form hydrogen bonds with urea, and Ile172, Phe93, Tyr80 make Hydrophobic contacts.[27] (Table S1).

We structurally aligned *Oryza sativa* Urease with 2R2X, 2I1J, and 2I1K (Figure 2). From Figure 2c, we can see the residues Gly121 and Phe93 that were present in the urea binding pocket of 2R2X were structurally aligned with the residues Asp622 and Phe580 of *Oryza sativa* urease, respectively. From Figure 2a, we can see only one of the residue (Asn6) present in the urea binding pocket of 2I1J was structurally aligned with Tyr350 of *Oryza sativa* Urease. From Figure 2b, we can see none of the residues present in the urea binding pocket of 2I1K were involved in structural alignment with the residues in *Oryza sativa* Urease.

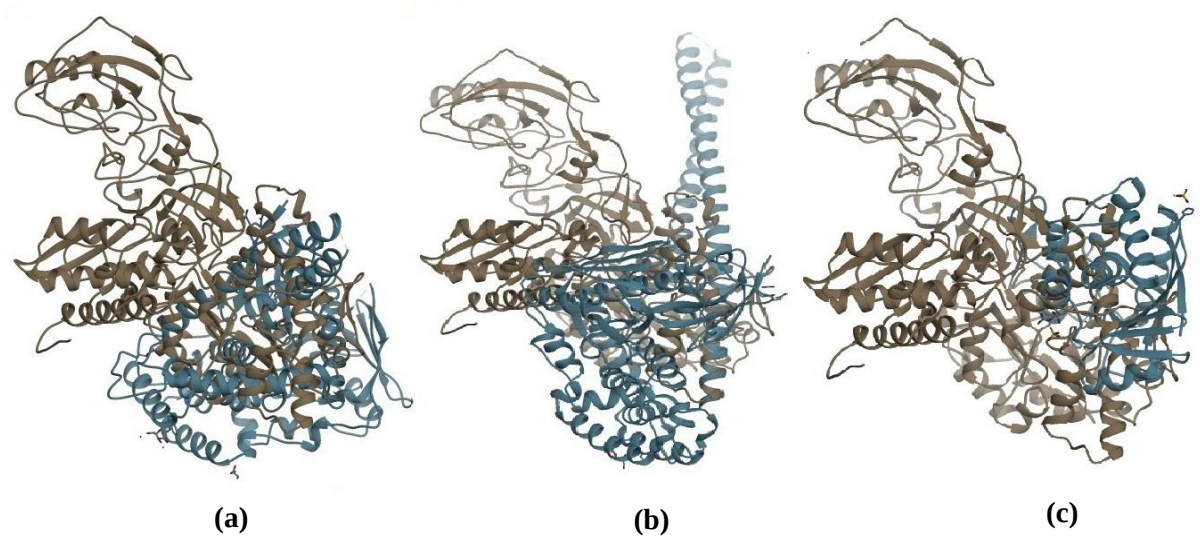

Figure 2. Structural superimposition of proteins with *Oryza sativa* Urease (copper) with (a) 2I1J_A (blue) (b) 2I1K_A (blue) (c) 2R2X_A (blue).

Since two residues were aligned in the case of 2R2X structural alignment (Table 1), we chose the corresponding structurally aligned amino acid residues, i.e., Asp622 and Phe580, as the urea binding site for *Oryza sativa* Urease (Figure 3).

Table 1. Structural alignment of amino acid residues of *Oryza sativa* urease with other urea bound proteins.

S.No.	PDB ID of Protein	Aligned Amino Acids	Corresponding amino acids of *Oryza sativa Urease*
1.	2R2X_A	GLY121	ASP622
		VAL81	Not Aligned
		PHE93	PHE580
2.	2I1K_A	ASN6	Not Aligned
		GLU73	Not Aligned
3.	2I1J_A	ASN6	TYR350
		GLU73	Not Aligned

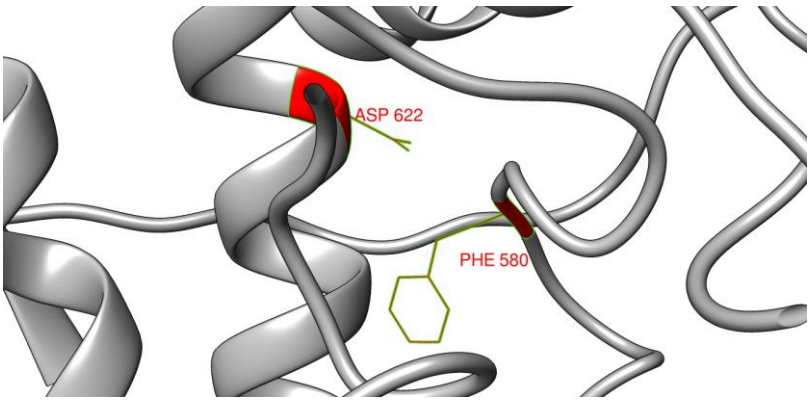

Figure 3. Predicted binding site in the modeled structure of *Oryza sativa* Urease, showing amino acid residues ASP622 and PHE580 in the binding pocket.

3.3. Generation of mutants.

To generate mutants, we initially found the amino acid residues that are present around the predicted substrate-binding pocket. The amino acid residues with a distance less than 3 Å from urea (present in the binding site) were Cys833, Glu621, Arg578, Asp622, and Thr577. The residues forming hydrogen bonds with urea (i.e., Phe580, Asp622) were exempted. All the 4 residues were checked for their possible amino acid replacements with the help of CUPSAT server. Thus, a list of possible stabilizing and destabilizing amino acid residues was found for each of the 4 amino acid point mutations in protein (Table S2). Hence, we obtained 2 single mutants and 2 double mutants- Arg578 was replaced with Cys, to generate OS_R587C, Arg578 replaced with Pro, to form OS_R578P. The double mutants produced were Arg578Val/Cys833Val (OS_R578V/OS_C833V) and Thr577Lys/Glu621Met (OS_T577K /OS_E621M).

Ligand (Urea) - protein (Urease) interactions for the native (originally modeled structure) and different mutants were analyzed by molecular docking to find mutant with the least binding energy, and thus, the highest binding affinity for urea. The native structure of modeled *Oryza sativa* Urease (OS_native) had binding energy of -4.47 kcal/mol and inhibition constant of 527.59 µM (Table 2), with Lys656, Ala619, Ile618, Phe580 being the interacting amino acid residues, forming hydrogen bonds with urea (Figure 4).

Table 2. Docking Analysis of *Oryza sativa* urease with urea.

S. No.	PROTEIN	Binding Energy (kcal/mol)	Inhibition Constant	Interacting Amino Acids (forming H-bonds)		Interacting Urea Atom	Distance (Å)
				Amino Acid	Atom		
1.	**OS_native**	**-4.47**	527.59 *µM*	LYS656	H	O	1.92
				ALA619	O	H	2.17
					O	H	2.14
				ILE618	O	H	2.14
					O	H	2.11
				PHE580	HN	O	2.30
2.	**OS_R578P**	**-5.13**	173.46 µM	GLU621	O	H	2.058
				ILE618	O	H	2.09
				ASP622	O	H	2.109
				ASP622	O	H	2.407
				ASP622	O	H	2.059
				ASP622	O	H	2.417
				THR577	O	H	2.23
3.	**OS_R578C**	**-5.28**	134.81 µM	ASP622	O	H	2.241
				ASP622	O	H	2.14
				THR577	O	H	2.0
				ILE618	O	H	1.86
				ASP622	O	H	2.16

S. No.	PROTEIN	Binding Energy (kcal/mol)	Inhibition Constant	Interacting Amino Acids (forming H-bonds)		Interacting Urea Atom	Distance (Å)
				Amino Acid	Atom		
				GLU621	O	H	2.129
4.	OS_R578V / OS_C833V	-5.21	150.88 µM	ASP622	O	H	2.213
				ASP622	O	H	2.166
				THR577	O	H	2.01
				ASP622	O	H	2.186
				ASP622	O	H	2.285
				ILE618	O	H	2.395
5.	OS_T577K / OS_E621M	-4.85	279.65 µM	GLU550	HN	O	2.132
				GLU550	O	H	2.0
				LYS577	H	N	2.04
				GLY551	HN	N	2.20
				HIS548	O	H	2.08
				THR574	O	H	2.278
				THR574	O	H	2.17

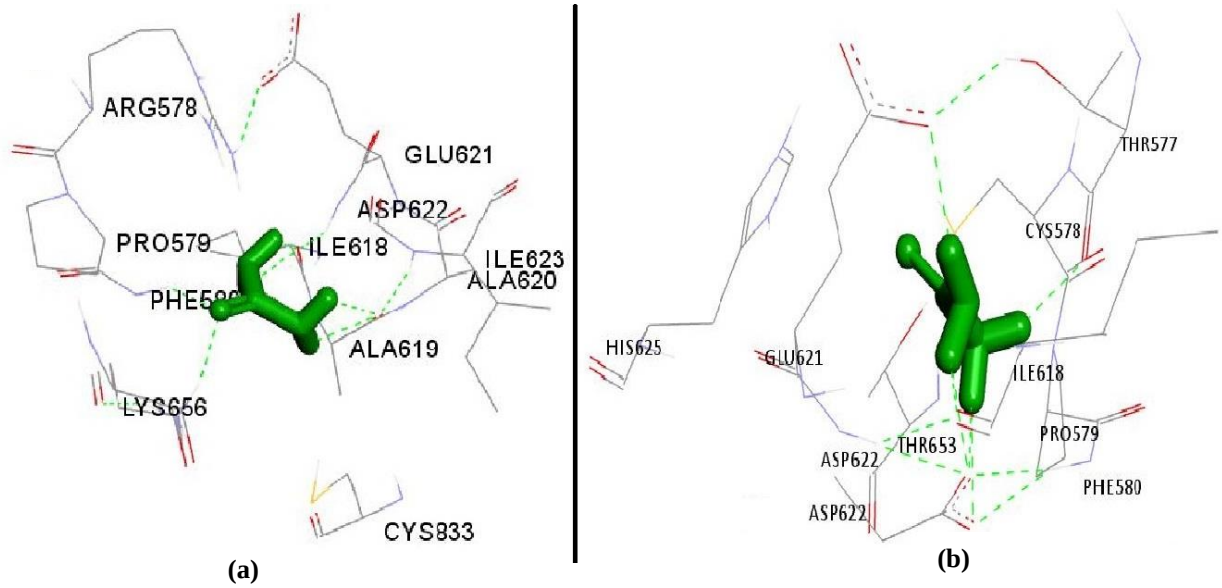

Figure 4. Computed structures are showing molecular interactions of **(a)** OS_native with urea and **(b)** OS_R578C with urea. Green color indicates hydrogen bonding.

Further, Asp622, Thr577, Ile618, Glu621 were the interacting amino acid residues of Arg578Cysmutant of *Oryza sativa* Urease (OS_R587C), with a binding energy of -5.28 kcal/mol and inhibition constant of 134.81 µM, thus regarded as best binding affinity with urea of the proteins given in Table 1. Arg578Val/Cys833Val (OS_R578V/OS_C833V) and urea gave second best binding affinity with a binding energy of -5.21, followed by mutant Arg578Pro (OS_R578P) and then mutant Thr577Lys/Glu621Met (OS_T577K /OS_E621M).

There have been many studies to determine urea-urease interactions or urease activity [28-40] of different plant species. The free binding energy of our mutated enzyme (-5.28 kcal/mol) is very less as compared to the energy predicted of *Arabidopsis thaliana (-3.28 kcal/mol),* and almost comparable to that of *Canavalia ensiformis* (-5.7 kcal/mol*)* [41]. In the recent past, we can see quite a number of in-silico studies in the literature that have been used to produce various genetically modified crops with novel properties, such as the production of β-carotene in rice endosperm [42], in-silico designing α-gliadin peptidase against celiac disease [43], developing crops that can withstand high abiotic stresses [44], etc. Thus, the computationally designed high urea-affinity enzyme can be used to generate environment

friendly, genetically modified rice crop which has minimum urea requirement, high Nitrogen Use Efficiency (NUE), without any compromise in its productivity.

4. Conclusions

We modeled the structure of *Oryza sativa* Urease, and it is found to be stable. We identified 2 urea binding sites in the modeled structure by analyzing the binding patterns of amino acids with urea in the 48 urea-binding proteins. We predicted the binding pocket consisting of Phe580 and Asp622 residues, based on high structural similarity and sequence alignment of native protein with Ricin. We created mutants of the modeled structure by replacing the amino acids near the binding site of the protein, with favorable and stabilizing amino acid residues. From the analysis of the mutants, we found OS_R578C (Arg578Cys mutation) to have higher urea binding affinity than the native protein. Furthermore, OS_R578P and double mutant OS_R578V/OS_C833V had similar binding energies, while OS_T577K/OS_E621M had the least binding affinity among the mutants, but still a higher affinity in comparison to the native protein. So, the mutant OS_R578C is stable and has the highest binding affinity for urea. Thus, a high affinity for urea will enhance urea metabolism and uptake by paddy, reducing environmental hazards caused by nitrogen losses from agricultural systems. This can be used as a template to generate an eco-friendly and stable genetically modified cereal crop (rice), which prevents hazards caused by excessive urea consumption in fields.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary files

Table S1. Amino acids involved in hydrogen bonding and hydrophobic contacts of closely related proteins with respect to Oryza sativa urease for urea ligand with the help of LIGPLOT (PDBsum).

PDB ID	Chain	Hydrogen Bonds (with urea)	Hydrophobic contacts (with urea)
2I1J	A	ASN6, GLU73	MET5, ASN74, PRO75, LEU76
2I1K	A	ASN6, GLU73	ASN74, MET5, LEU76
2R2X	A	GLY121, VAL81	ILE172, PHE93, TYR80

Table S2. List of stabilizing and destabilizing amino acids for the mutation in *Oryza sativa* Urease using CUPSAT server.

Amino Acid	Overall Stability	
	Stabilizing Amino Acids	Destabilizing Amino Acids
CYS833	VAL	GLY,ALA,LEU,ILE,MET,PRO,TRP,SER,THR,PHE,GLN,LYS,TYR,ASN,GLU,ASP,ARG,HIS,CYS
GLU621	ALA,VAL,LEU,ILE,MET	GLY,PRO,TRP,SER,THR,PHE,GLN,LYS,TYR,ASN,GLU,ASP,ARG,HIS,CYS
ARG578	VAL,LEU,ILE,MET,PRO,TRP,LYS,CYS	GLY,ALA,SER,THR,PHE,GLN,TYR,ASN,GLU,ASP,ARG,HIS
ASP622	ALA,MET,PRO,TRP,TYR	GLY,VAL,LEU,ILE,SER,THR,PHE,GLN,LYS,ASN,GLU,ASP,ARG,HIS,CYS
THR577	ALA,PRO,GLN,LYS	GLY,VAL,LEU,ILE,MET,TRP,SER,THR,PHE,TYR,ASN,GLU,ASP,ARG,HIS,CYS

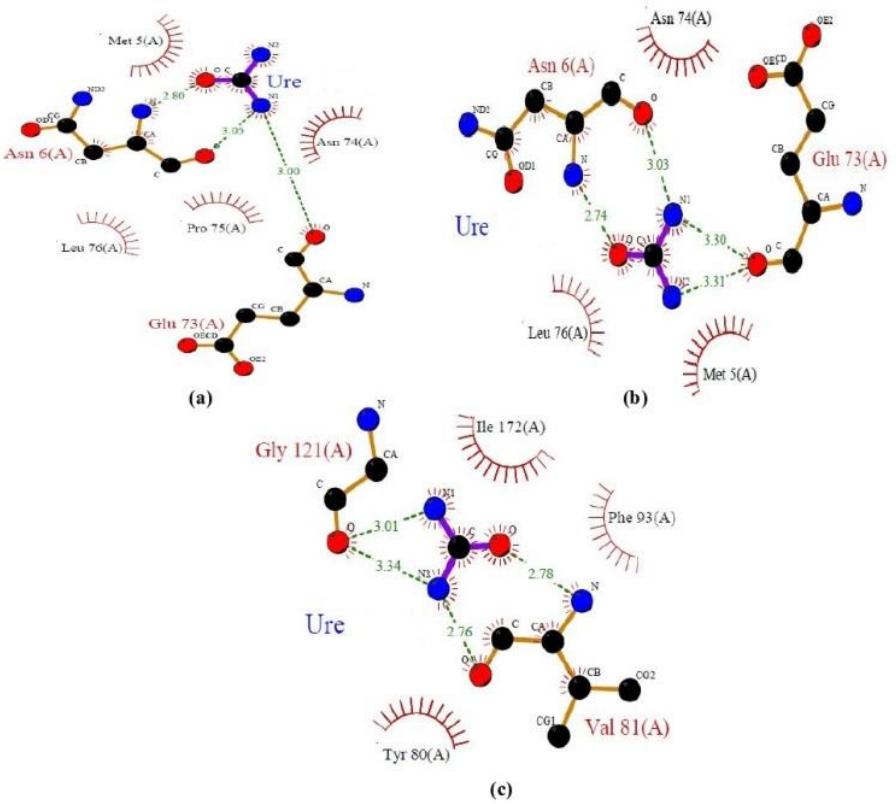

Figure S1. LIGPLOTs showing the interacting residues of a protein molecule with urea (a) Interaction of urea with chain A of 2I1J, ASN6, GLU76 are forming Hydrogen bond, and MET5, ASN74, PRO75, LEU76 have hydrophobic contacts with urea (b) with chain A of 2I1K, ASN6, GLU73 are forming hydrogen bonding and ASN74, MET5, LEU76 have hydrophobic contact with urea (c) with chain A of 2R2X, GLY121, VAL81 are forming hydrogen bonding and ILE172, PHE93, TYR80 have hydrophobic contacts with urea.