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RESEARCH

Studies on Deimmunization of Antileukaemic L-Asparaginase to have Reduced Clinical Immunogenicity- An *in silico* Approach

L. N. Ramya · Krishna Kanth Pulicherla

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Abstract Protein therapeutics, particularly of heterologous origin are shown to elicit immunogenic responses which result in adverse allergic reactions in spite of their promising clinical benefit. L-Asparaginase is one such well known chemotherapeutic agent that has enhanced the survival rates to 90 % in the treatment of acute lymphoblastic leukaemia for past 30 years. But the use of this enzyme is accompanied by hypersensitive reactions ranging from allergy to anaphylactic shock which have a drastic influence in treatment outcomes. Numerous attempts have been made to minimize the problems of immunogenicity, which remained as a major bottleneck in the treatment protocols. Conjugating the enzyme L- Asparaginase with PEG was successful as it has reduced the complications in therapy and frequency of injections (dosages), and thus became prominent in reducing the immunogenicity up to a certain extent. Keeping the bottlenecks in consideration during the development of therapeutics, the present study concentrates on engineering of protein as an alternative to the PEGylated enzyme, having reduced immunogenicity as an inbuilt character of protein by using *in silico* approaches. L-Asparaginase from *Escherichia coli* and *Pectobacterium carotovorum* were selected for the present study. The methodology consists of (i) locating the B and CD4+ T cell epitopes of enzyme by *in silico* tools (ii) generating point mutations of these epitopes to alter or reduce the immunogenicity of protein (iii) generating enzyme models by molecular modelling (iv) assessing the binding affinity of the substrate with L-Asparaginase variants by *in silico* docking methods using Autodock 4.2 and (v) validating the mutated model for stability by molecular dynamics simulation studies using Gromacs.

Keywords L- Asparaginase · Hypersensitive reactions · Neutralizing antibodies · Anaphylactic shock · *Pectobacterium carotovorum* · Immunogenicity · Acute lymphoblastic leukaemia

L. N. Ramya
Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur 522510, A.P., India
e-mail: nagramya@gmail.com

K. K. Pulicherla (✉)
Center for Bioseparation Technology, VIT University, Vellore 632014, TN, India
e-mail: kkpulicherla@gmail.com

Introduction

Enzymes are widely used as therapeutics for around 40 years [1]. L- Asparaginases (L-Asparagine amidohydrolase; EC 3.5.1.1) are the enzymatic therapeutics that have been used to treat childhood acute lymphoblastic leukaemia (ALL) for the past few decades [2, 3] and were reported to show enhanced cure rates or survival rates. These enzymes are involved in catalyzing the hydrolysis of L-Asparagine into aspartic acid and ammonia. Tumour cells are in great need of L- Asparagine for their rapid proliferation and depend on external supply of L- Asparagine for their growth. So, by administering L-Asparaginase, the tumour cells are made to starve of that particular amino acid, which leads to their apoptosis [4, 5]. Three types of L- Asparaginase preparations are available in the market, namely native and PEGylated forms (PEG-Asparaginase) from *Escherichia coli* and enzyme from *Erwinia* species (Erwinase). All the forms of the enzyme have similar functionality but differ with respect to pharmacokinetic properties [6]. Like all large proteins, L-Asparaginase was also found to elicit immune responses that lead to the production of anti-asparaginase neutralizing and non neutralizing

antibodies which is the main cause of adverse reactions. This leads to a reduction in the concentration of L- Asparaginase enzyme in the body, causing ineffective depletion of L- Asparagine [7, 8]. The production of these antibodies during the therapy causes hypersensitive problems ranging from allergy to anaphylactic shock which affects the treatment outcomes [9, 10]. One of the most successful attempts in attending the problem of clinical immunogenicity is conjugating the drugs

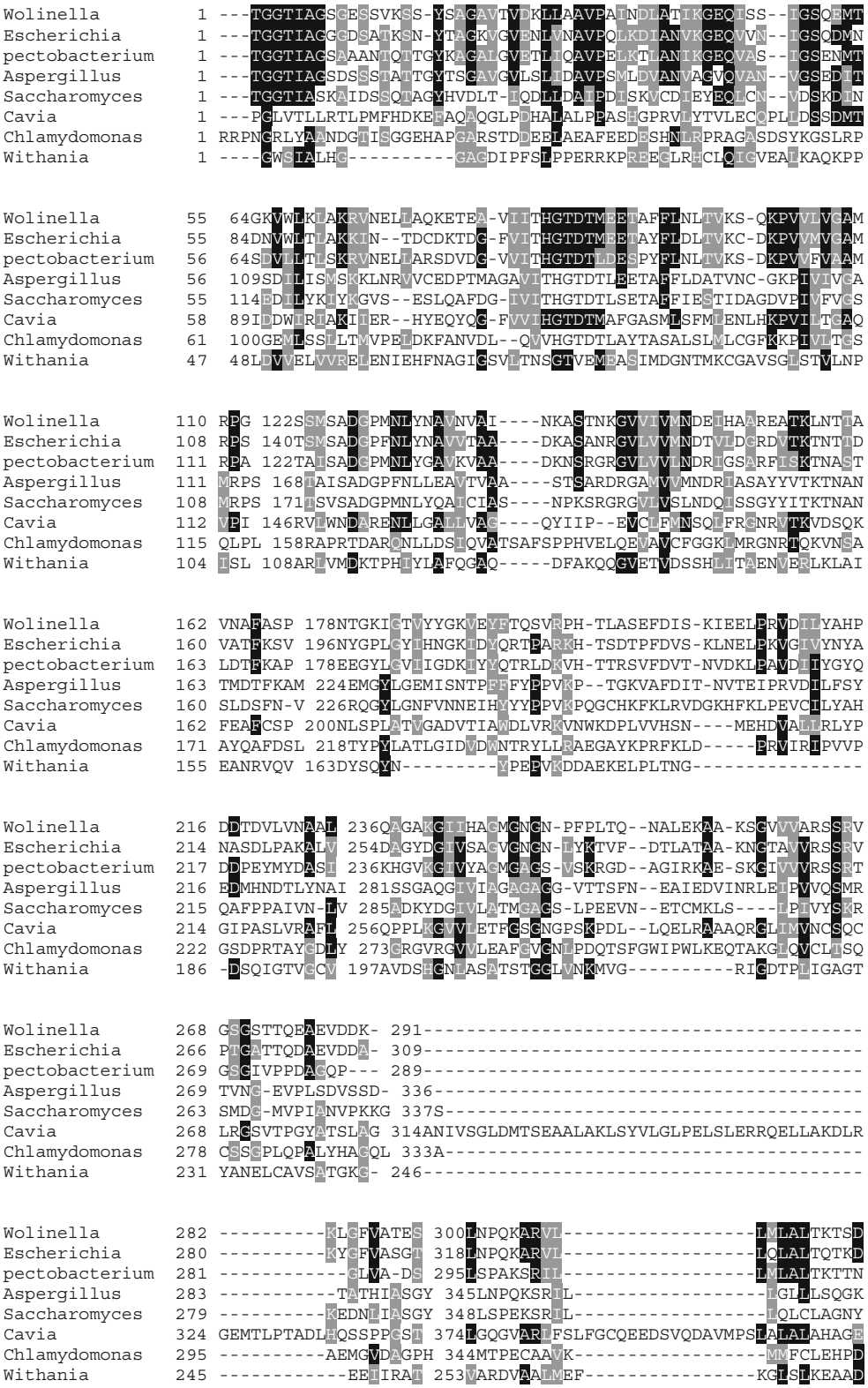

Fig. 1 Multiple sequence alignment of L-Asparaginase sequences showing maximum conservation in between 57 and 373 amino acids

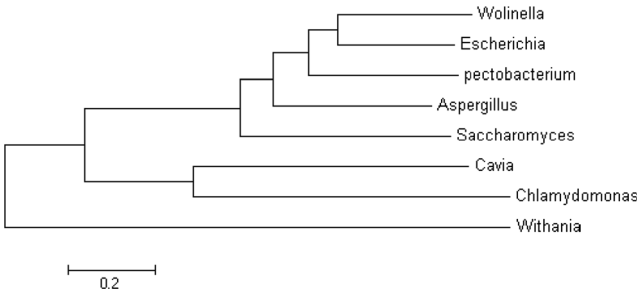

Fig. 2 Phylogenetic tree of L-Asparaginase sequences from different organisms constructed by the maximum parsimony method

with polyethylene glycol (PEG). An initial attempt has been made in the case of L-Asparaginase enzyme where it was successfully coupled with polyethylene glycol [10, 11] which has enhanced the persistence of enzyme in circulation and increased the stability of the enzyme by protecting from proteolysis. Immunogenicity of PEG Asparaginase (4–8 %) is relatively low showing less allergic reactions when compared to the native enzyme from *Escherichia coli* and so it is used in second- line treatment nowadays in some treatment protocols [7, 12–15]. But PEG is not the primary choice in the treatments mainly because of the increase in size as it binds to water molecules leading to reduced drug efficacy. And also as PEG is obtained by the process of chemical synthesis, it shows polydisperse property and also causes many complications as it accumulates in liver due to its high molecular weight [16].

Later, this problem was solved by substituting crisantaspase (Erwinase) in the place of both native *Escherichia coli* asparaginase and PEGylated forms [6, 8]. But *Erwinase* has the problems of shorter half-life when compared with preparations from *Escherichia coli* and also reported to have a more glutaminase side activity which is the reason behind most of the side effects in treatment protocols [2, 3]. Due to such problems, enzymes of heterologous property are not advisable for humans in cases of long term or repeated usage in the treatment protocols.

Enzyme from *Pectobacterium carotovorum* has been selected and used in the present study as it has the properties that are much better when compared with the enzyme sourced from *Escherichia coli* and *Erwinia chrysanthemi* that include less glutaminase side activity leading to reduced side effects and also have the ability of hydrolyzing β-aspartyl peptides. Recently, attempts were also made to enhance the production of glutaminase free L- Asparaginase from *Pectobacterium carotovorum* [17, 18]. But like every therapeutic protein, this bacterial enzyme was also observed to have a bottleneck in the form of immunogenicity problems and hence we made an attempt in the present study to reduce the protein immunogenicity by an *in silico* approach of identifying and mutating epitopic regions of L-Asparaginase from *Pectobacterium carotovorum* (PecA) and also the FDA approved L-Asparaginase from *Escherichia coli* (EcA) as model enzymes.

Materials and Methods

Retrieval of L- Asparaginase Protein Sequences and Construction of Dendrogram

L-Asparaginase sequences was considered from eight tremendous producers of enzymes such as *Aspergillus niger, Cavia porcellus, Withania somnifera, Wolinella succinogenes, Saccharomyces cerevisiae, Chlamydomonas reinhardtii, Escherichia coli* and *Pectobacterium carotovorum* to study about sequence conservation and also to understand about the phylogenetic evolutionary relation. The sequences were obtained in the form of FASTA format from the biological database - National Centre for Biotechnology Information (NCBI). Multiple sequence alignment of all the above collected sequences from different domains of living beings was done using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). The parameters considered here include a gap

Table 1 Regions of conserved motifs for enzyme L- Asparaginase from different sources

S. No	Organism	Motif 1	Motif 2	Motif 3	Motif 4	Motif 5	Motif 6
1.	*Chlamydomonas reinhardtii*	147–170	123–143	188–227	–	–	–
2.	*Cavia porcellus*	135–158	111–131	170–209	–	–	–
3.	*Aspergillus niger*	157–180	134–154	194–233	72–121	235–271	346–375
4.	*Pectobacterium carotovorum*	111–134	88–108	148–187	27–76	190–226	296–325
5.	*Escherichia coli*	107–130	84–104	144–183	25–74	186–222	297–326
6.	*Wolinella succinogenes*	88–137	148–187	32–76	295–330	194–222	3–17
7.	*Saccharomyces cerevisiae*	117–166	177–216	61–105	–	222–250	32–46
8.	*Withania somnifera*	78–106	131–145	31–59	165–176	113–120	84–91

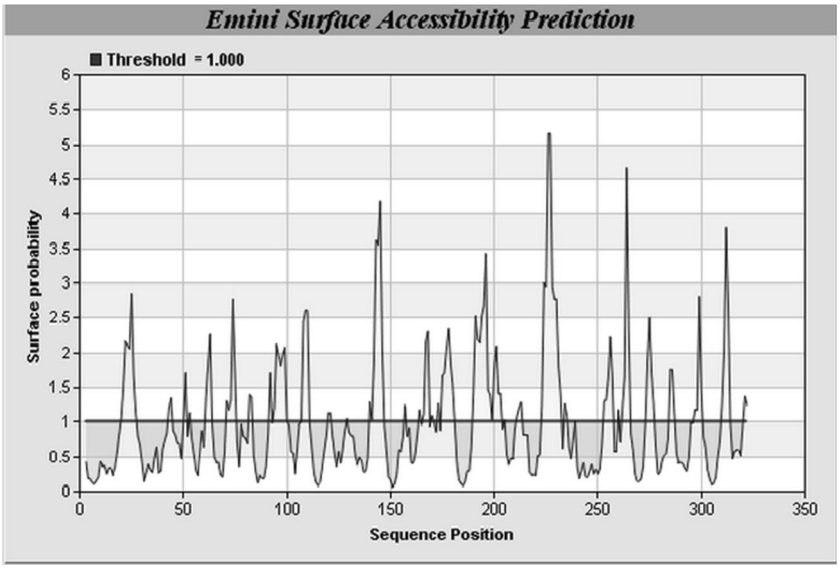

Fig. 3 Graph showing maximum antigenicity at 226–232 in PecA

open of 10, gap extension of 0.20 and gap distances of 5 with no end gaps.

All the eight sequences of enzyme L- Asparaginase were considered in the present work to study about their evolutionary relationships with the help of MEGA 5.1 (Molecular evolutionary genetic analysis). Phylogenetic tree was constructed from all the eight sequences using Maximum parsimony method [19].

Identification of Conserved Motifs and Domains

MEME (Multiple EM for Motif EliCitation) [20] is a well known tool which allows to discover motifs in protein or DNA sequences. Input parameters such as width of the motif and maximum number of motifs were specified. This tool is helpful to perform motif-motif database search, motif-sequence database search and discovery of motifs etc. Blocks are also displayed where the start and end point of the motifs were displayed. Location of motifs or sites obtained in the MEME describes the conserved regions that are related to the structural and functional properties of the enzyme in the process of evolution.

Pfam is a database which is used to analyze the domains of L- Asparaginase where the sequences were submitted to Pfam in the form of UniProt number. All the sequences were verified for the domain organization related to L- Asparaginase protein [21].

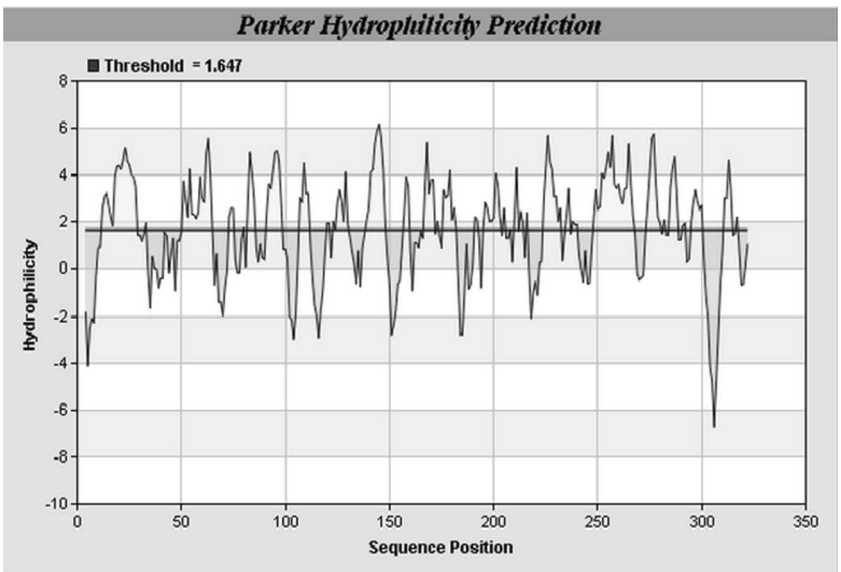

Fig. 4 Graph showing maximum antigenicity at 145–151 in PecA

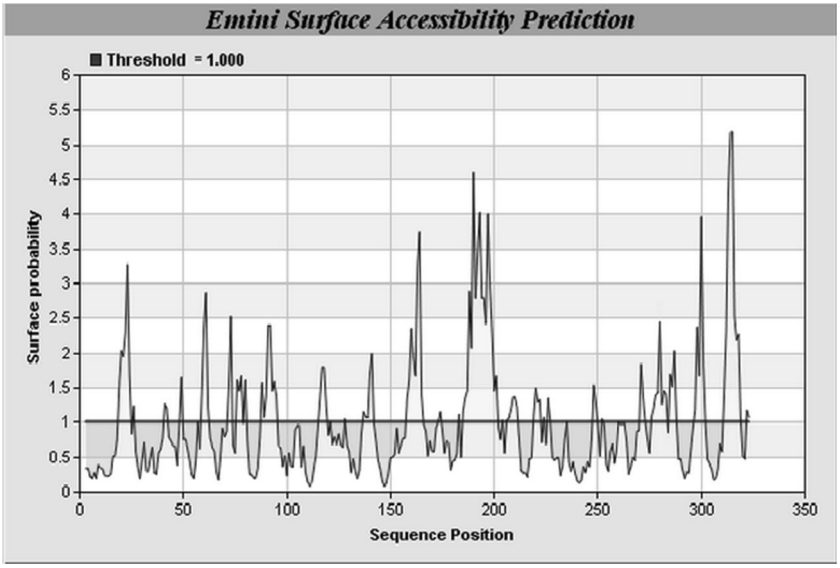

Fig. 5 Graph showing maximum antigenicity at 312–317 in EcA

Identification of Antigenic Epitopes of L-Asparaginase

Sequences of enzymatic drug L- Asparaginase sourced from EcA and PecA were taken from NCBI and submitted to IEDB analysis (www.tools.immuneepitope.org) resource [22] to determine the B-cell and T-cell epitopic peptides. The sequences were primarily submitted for B-cell epitope prediction based on the principle of surface accessibility and hydrophilicity [23–25]. Two sequences, i.e., EcA and PecA were scanned for identification of putative T-cell epitopic sites with the help of ALL-associated HLA-DR DRB1*0401 allele [26] using the consensus methodology.

In silico Mutagenesis and Molecular Modeling

Based on the scores obtained for B and T cell epitopes of L-Asparaginase from both sources (PecA and EcA), peptides having more antigenic score were selected as targets for *in silico* engineering, as they may be the major contributors for clinical immunogenicity. Accordingly, *in silico* site directed mutations were carried out for the enzyme by the software pymol. The generated mutants were analyzed by SAVS and were validated using Ramachandran plot and the structures were further used for docking studies to analyze the impact of these mutations on the primary catalytic activity of the antileukemic L-Asparaginase.

Building Ligand Structures

Ligand structure L- Asparagine were drawn using Hyperchem software. Amino acids were selected from the database menu of the software to draw 2-dimensional structures and are modeled into 3D structures. Energy minimization for the ligand structures were also done by using hyperchem through

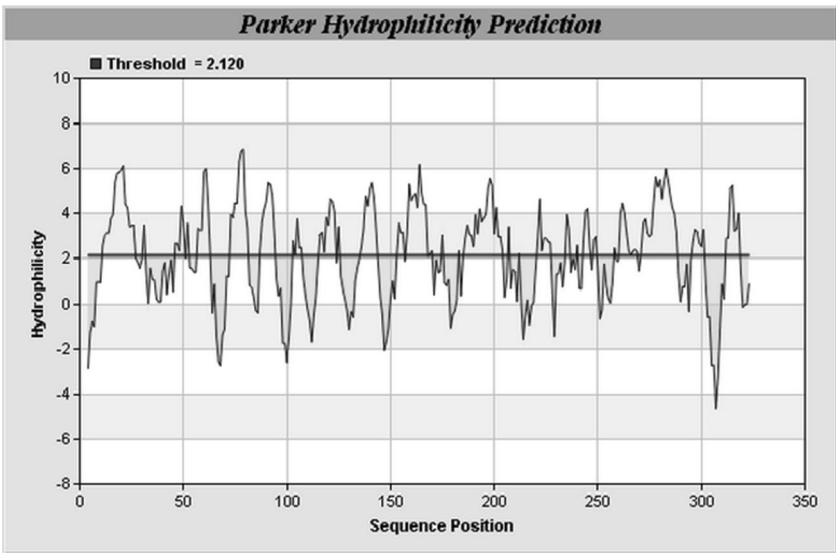

Fig. 6 Graph showing maximum antigenicity at 76–82 in EcA

Table 2 B-cell epitopic scores obtained for EcA and PecA L-asparaginase

	Method	Peptide	Region	Score
Escherichia coli	Surface accessibility	QTCDPQ	312–317	1.462
	Hydrophilicity	DCLKTDG	76–82	4.114
Pectobacterium carotovorum	Surface accessibility	YQDDPEY	226–232	5.167
	Hydrophilicity	DKNSRGR	145–151	6.186

semi-empirical and conjugate gradient Polak- Ribiere methods to obtain the most stable geometries which are further used for docking studies.

Molecular Docking Studies of L-Asparaginase

Molecular Docking studies for PecA and EcA were performed using Autodock 4.2 version (http://autodock.scripps.edu/resources/adt) using the free energy function and the Lamarckian genetic algorithm [27–29]. Polar hydrogens were added to the protein and gasteiger charges were added to the ligand. Gpf (grid parameter file) and Dpf (docking parameter file) files were prepared and the grid points for autogrid calculations were set with active site residues in the center of the grid box with a grid spacing of 0.375 A^0 -1 A^0. The parameters of docking include 50 randomly placed individuals with 15 million energy evaluations and a maximum of 27,000 generations with a mutation rate of 0.02, over a cross rate of 0.80 values. Independent dockings of one hundred were carried out for the ligand where the result was represented in terms of binding energy. Out of 100 docked conformations obtained, the structure or protein- ligand complex with best docking energy (lowest docked energy) was selected. Clustering histogram gives the detained data of the interactions computed at specific RMSD (Root mean square deviation). Overall docking energy of a molecule in Autodock is expressed as the sum of intermolecular interaction energies that includes van der Waals attractive and repulsive energies, H-bonding, electrostatic energy and the total internal energy [30].

Table 3 Best Antigenic scores of T-cell epitopes after mutating with all the amino acids

S No	Amino acids	*Escherichia coli*	*Pectobacterium carotovorum*
1.	Native	0.96	0.19
2.	Alanine	2.49	19.24
3.	Valine	2.04	3.15
4.	Leucine	1.68	2.57
5.	Proline	6.35	24.75
6.	Glycine	3.46	24.75
7.	Serine	2.96	24.75
8.	Isoleucine	1.40	1.49
9.	Methionine	2.02	4.48
10.	Cysteine	3.98	5.39
11.	Phenyl Alanine	2.76	1.98
12.	Tyrosine	5.86	3.01
13.	Histidine	3.32	19.54
14.	Lysine	5.04	24.75
15.	Asparagine	3.12	24.75
16.	Glutamine	5.28	24.75
17.	Threonine	2.49	15.70
18.	Glutamic acid	5.07	24.75
19.	Aspartic acid	5.13	24.75

Molecular Dynamics Simulation Studies

L- Asparaginase variants with mutated T-cell and B-cell epitope from both *Pectobacterium carotovorum* and *Escherichia coli* has been subjected to MD simulation studies. Molecular dynamics (MD) simulations were performed using the GROMACS 4.5.5 software with the standard GROMOS96 force field [31, 32]. GROMOS96 force field was selected for the system as this is embedded in the software itself and also it calculates both bonded and non-bonded parameters [33]. Coordinate files and Topology files related to the ligand were generated using the PRODRG program [34]. Protein is filled with a cubic system with solvent (water) and is neutralized by adding counter ions by replacing water molecules, respectively. Protein complex with water is subjected to energy minimization using the steepest descent approach realized in the GROMACS package for getting lowest energy conformations. Both temperature and pressure coupling steps were carried out, followed by 15 NS, MD simulations using the periodic boundary conditions in all three dimensions [35]. All these processes were carried out on the Vega cluster with the help of message passing interface implementation (MPI).

Table 4 T-cell epitopic scores of different sources producing asparaginase

S No	Organism	Region	Score	Sequence
1.	*Escherichia coli*	23–37	0.84	QQELRYIEALSAIVE
2.	*Pectobacterium carotovorum*	227–241	0.19	DPEYMYDASIKHGVK
3.	*Withania somnifera*	108–122	1.69	LARLVMDKTPHIYLA
4.	*Aspergillus niger*	36–50	2.36	NGLNFTQMNTTLPNV
5.	*Guinea Pig Serum*	32–46	1.12	GPGLVTLLRTLPMFH
6.	*Chlamydomonas reinhardti*	139–153	0.88	SAAYQAFDSLTYPYL
7.	*Withania somnifera*	38–52	1.69	LARLVMDKTPHIYLA
8.	*Saccharomyces cerevisiae*	56–70	0.22	EETAFFLDLTINSEK

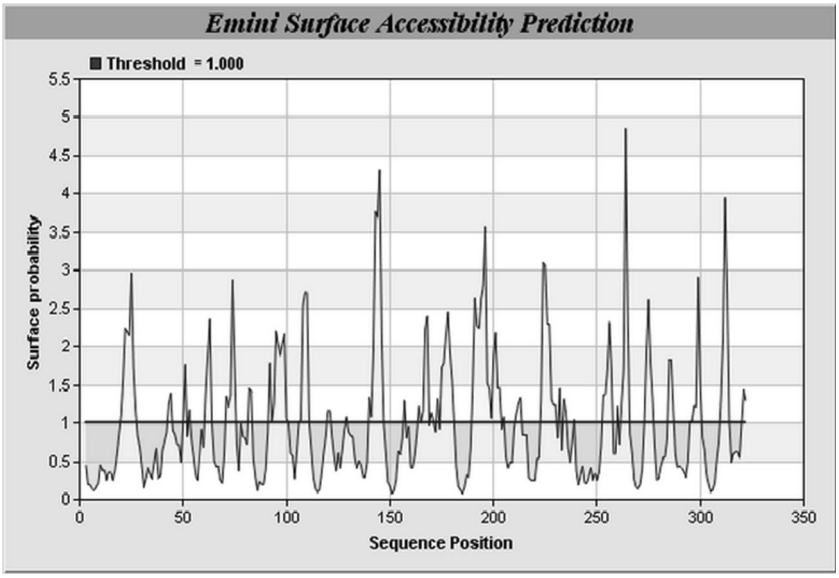

Fig. 7 Graph showing antigenicity score for mutated protein at 226–232 in PecA

Results and Discussion

Multiple Sequence Alignment and Construction of a Phylogenetic Tree

Conserved amino acids in all the eight sequences were obtained and are represented by boxshade server (Fig. 1). The antigenic determinants are characteristics of surface regions and are found in places where polar hydrophilic amino acids are present in huge number. Previous studies reported that the presence of more aromatic amino acids like tyrosine, histidine in a protein may render antigenic property of the protein L-Asparaginase which is in agreement with the results obtained in this study. Also, from the result, it was observed that polar uncharged residues like threonine, serine, histidine might be responsible for antigenically active regions of a protein. In case of asparaginase from Wolinella (D217), Pectobacterium (D218), Aspergillus (D217) and Withania (187), D218 of epitopic peptide YQD**D**PEY is highly conserved whereas Alanine is present in E. coli (A215) and Saccharomyces (A216). Ile and Ser are found in Cavia (I215) and Chlamydomonas (S223) in the place of D218 which clearly shows that antigenic property might be influenced. In another epitopic peptide, DKN**S**RGR, Serine was found to be conserved in Wolinella (S138), E.coli (S145), Pectobacterium (S148) and Aspergillus (S144) whereas same place is occupied by lysine, Isoleucine, Histidine and Alanine in Saccharomyces (K140), Cavia (I145), Chlamydomonas (H147) and Withania (A136) respectively. From the ClustalW result, it was observed that maximum similarity and identity was observed to be in between Pectobacterium, Aspergillus, *Escherichia coli* and Wolinella. The relationship between prokaryotic and eukaryotic

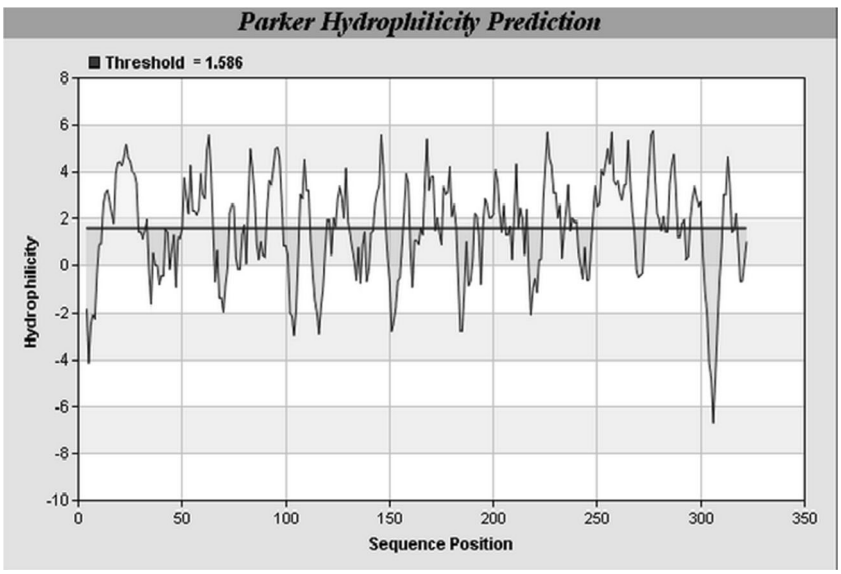

Fig. 8 Graph showing antigenicity score for mutated protein at 145–151 in PecA

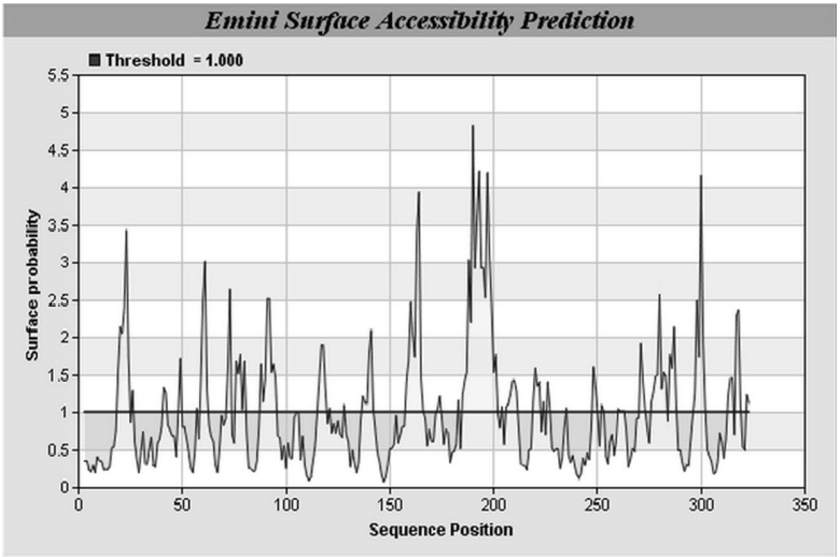

Fig. 9 Graph showing antigenicity score for mutated protein at 312–317 in EcA

producers of L- Asparaginase was examined by constructing a dendrogram using Maximum parsimony method. Dendrogram revealed that all the domains of living beings ie., plants, animals, bacteria, fungi and algae appeared in different clusters. Fungal sources *Aspergillus* and *Saccharomyces* appeared in same clusters. *Pectobacterium* was found to be appearing in different cluster, but nearer to *Aspergillus* and *Saccharomyces*. Bacterial species *Wolinella and Escherichia coli* appeared in same cluster showing sequence level similarity. The plant source, *Withania* appeared in different cluster separately from all the others. Enzyme from plant sources was not in wide clinical usage as the problem of antigenicity was very high due to the differences that exist in terms of pharmacological properties. *Cavia porcellus* shows sequence similarity with eukaryotic algae *Chlamydomonas* and so appeared under similar cluster showing sequence level similarity (Fig. 2). For the first time, in the year 1922, Clementi identified that, *Cavia porcellus* (Guinea pig serum) is a rich source of the enzyme L- Asparaginase [36] and also it was reported to have nil glutaminase side activity, but as the production of the enzyme is insufficient when compared with other sources, this was not in clinical usage [2, 37, 38]. Other species like *Wollinella succinogenes* were also found to have L- Asparaginase that act on lymphomas, but did not gain importance for clinical use [39, 40].

Conservation of Motifs

The MEME motif algorithm uses a Bayesian probabilistic model for searching the motifs for all the sequences and optimizes the statistical parameters by Expectation Maximization algorithm [20]. A total of six motifs labelled as 1–6 were identified by using the tool MEME (Motif-based sequence analysis tool). Distribution of these motifs (regions) among eight L- Asparaginase

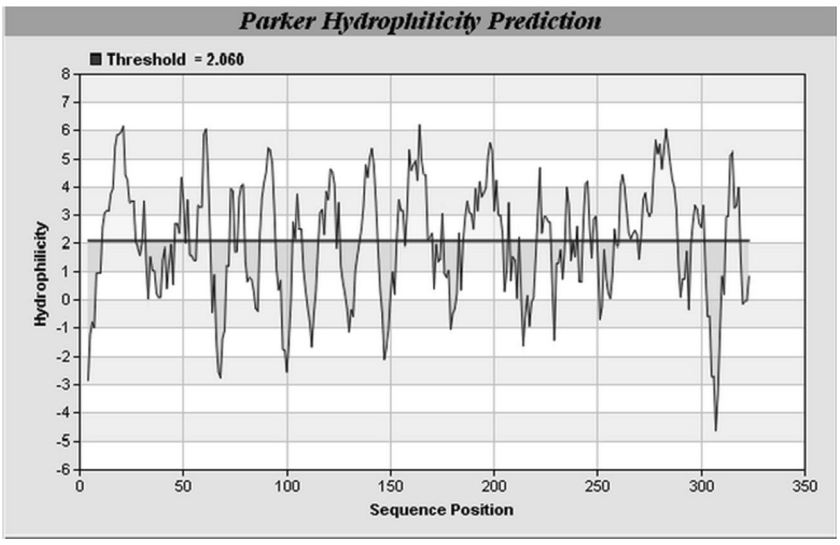

Fig. 10 Graph showing antigenicity score for mutated protein at 76–82 in EcA

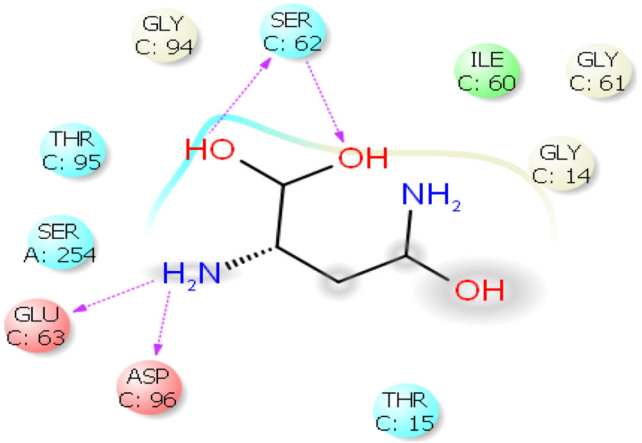

Fig. 11 Active site interactions of L-Asparaginase from *Pectobacterium carotovorum* with asparagine bound in it

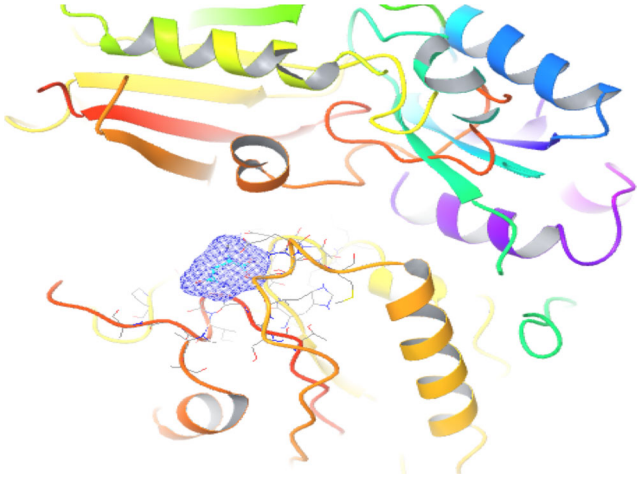

Fig. 13 Enzyme L-Asparaginase with substrate asparagine in the active site (Mesh)

sequences shows 3 motifs in common. Motif 5 and 6 have been observed to be present in only *Aspergillus niger*, *Pectobacterium* and *Escherichia coli* (Table 1). This clearly shows that the major function of the enzyme in spite of its origin from different sources was conserved and these motifs clearly suggest their possible role in structural and enzymatic functions.

The domain analysis using Pfam clearly revealed that the sequences from various sources of the enzyme L- Asparaginase have a single domain organization ranging from 25 to 340 that belong to L- Asparagine amidohydrolase but have slight differences with respect to the number of amino acids.

Identification of Antigenic Epitopes of L-Asparaginase

L- Asparaginase sequences of EcA and PecA were taken in FASTA format and submitted to the IEDB prediction server to determine the antigenic epitopes in the enzyme. Antigenic sites in a protein are formed either by continuous sequential regions or by several antigenic determinant groups. In the process of reducing the immunogenicity of the enzyme drug L- Asparaginase, primarily, B- cell epitopic regions of the

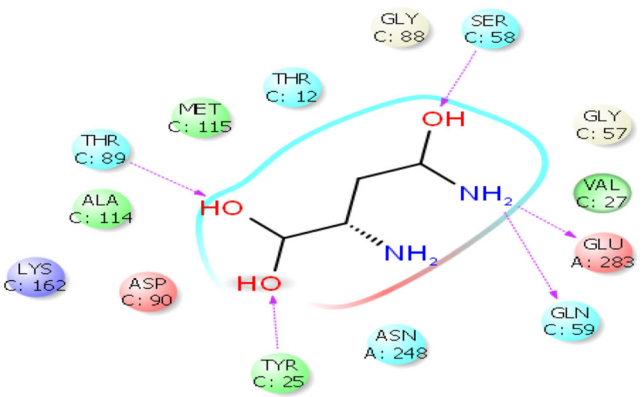

Fig. 12 Active site interactions of L-Asparaginase from *Escherichia coli* with asparagine bound in it

protein were predicted by continuous method based on the major antigenic properties like Emini surface accessibility method which is based on surface accessibility scale and also by the Parker hydrophilicity method as most of the antigenic proteins are rich of charged and polar residues along with lacking in hydrophobic residues. B-cell epitopic sites from PecA were identified which have two peptide regions, namely ^{226}YQDDPEY^{232} (Fig. 3) and ^{145}DKNSRGR^{151} (Fig. 4) whereas for EcA, the regions were found to be ^{312}QTKDPQ^{317} (Fig. 5) and ^{76}DCDKTDG^{82} (Fig. 6). More is the hydrophilic part in the epitopic site of protein, more is the immunogenicity. Thus replacement of one of the residues of epitopic sites with valine and leucine which are hydrophobic in nature has led to a reduction in the antigenic scores in an attempt made by Bander for ^{177}Lutetium-Labeled J591, a Monoclonal Antibody to Prostate-Specific Membrane Antigen [41] (Table 2).

Activation of CD4+ cells is an important step in the development of immunity against foreign bodies. Crucial step in the process is the recognition of epitope that is presented in complex with the help of MHC II molecules on the surface of antigen presenting cells. Mutating or removal of T-cell epitopes helps in attaining success related to immunogenicity when it does not affect the native catalytic property of protein [42–44]. To identify various peptide segments related to T-cell epitopes, one of the widely used methods is based on the consensus algorithm [45, 46]. So, T-cell epitopes for both PecA and EcA proteins having key residues which are important for MHC binding were identified by giving an input parameter of allele DRB1*0401 that is specific for ALL based on the consensus method of IEDB. Peptide of 15mer of both PecA and EcA were obtained which cover the regions of ^{227}DPEYMYDASIKHGVK^{241} and ^{301}KARVLLQLALTQTKD^{315} with a lower percentile of 0.19 and 0.96 (Table 3). Eukaryotic enzymes, particularly sourced from Chlamydomonas and Saccharomyces were also checked for immunogenicity scores based on MHC binding (Table 4).

Table 5 Molecular docking energy level table for EcA and PecA

S. No	Organism	Est. free energy of binding (Kcals/mole)	Number of H-bonds formed in interaction	vdW + Hbond + desolv Energy (Kcals/mole)	Electrostatic energy (Kcals/mole)	Total intermolec. energy (Kcals/mole)
1.	*E.coli* (Native)	−11.86	7	−2.56	−1.56	−7.03
2.	*E.coli* (Mutated)	−12.01	7	−2.64	−1.24	−6.53
3.	*Pectobacterium carotovorum* (Native)	−10.26	4	−1.00	−0.34	−7.42
4.	*Pectobacterium carotovorum* (Mutated)	−10.24	5	−1.33	−1.03	−7.94

In silico Mutagenesis

Identified antigenic epitopes of L- Asparaginase from PecA and EcA were mutated in the regions of B- cell epitopes and T-cell epitopes by the help of pymol software. The obtained enzyme mutant structures were then analyzed by using SAVS and validated by using Ramachandran plot. In the present study, for EcA, in the case of B- cell epitopes, promising scores ie., reduced antigenicity were obtained in case of K314C (1.462) (Fig. 7) and D78L (4.114) mutations (Fig. 8) whereas for PecA, 2.299 were obtained in E231V mutation (Fig. 9) and a score of 3.443 were obtained in D145L mutation (Fig. 10). In the case of T-cell epitopes, Q307P in EcA was found to be the best replacement, whereas in case of PecA, mutations D229G showed promising results with respect to the reduced antigenic property. It was observed that these replacements would less likely influence the structure of the enzyme or its functional properties and further studies are carried out towards evaluating the advantages of antigenically modified enzyme drugs in therapy. Zainab et al. in 1994 made a trial for reducing immunogenicity by site directed mutagenesis in case of *Erwinia chrysanthemi* where it was observed that replacement of any other amino acid in the place of proline, which is the most important amino acid for antibody binding in the epitopic region was found to reduce the immunogenicity. Particularly, change of proline to threonine (P285T) has led to a reduction in the immunogenicity of the enzyme [46]. Alanine scanning was done in the case of *E.coli* L- Asparaginase wherein most prominent amino acids responsible for antigenicity were identified. Alanine was placed instead of three continuous alkaline residues where mutation 195RKH197 to 195AAA197 reduced antigenicity greatly [47]. Based on the scores obtained after mutations, final validated structures were selected and further subjected to docking studies to study about the functional aspects of the protein.

Table 6 Binding energies of native and mutated L-asparaginase after docking

S. No	Organism		ASN (KCals/mole)
1.	*Escherichia coli*	Normal	−11.86
		Mutated	−12.01
2.	*Pectobacterium carotovorum*	Normal	−10.26
		Mutated	−11.60

Determination of Primary Catalytic Efficiency of L- Asparaginase and its Variants by Docking Studies

The substrate L- Asparagine was drawn using the software hyperchem and the structures were subjected to energy minimization to rectify the errors attained by crystallization. Energy minimization scores of L- Asparagine was found to be −1767.2177 KCal/mole. PDB structures of EcA and PecA were taken from Protein Data Bank having the PDB IDs 3ECA and 2JK0 respectively. Both the native and mutated proteins were subjected to flexible docking, giving Gly14, Thr15, Gly61, Ser62, Glu63, Gly94, Thr95, Asp96, and Ala120 as active site flexible residues [37, 48] (Fig. 11) for PecA and Thr12, Ser58, Gln59, Thr89 and Asp90 for EcA [49] (Fig. 12). Enzymes were docked against the substrate L- Asparagine (Fig. 13) and the binding energies were calculated. Docking studies give a clustering histogram for 100 conformations along with their RMSD values. The lowest binding energy values with an RMSD of 0.00 A^0 were taken from the most populated cluster. Hydrogen bonds, van der Waal's forces and total internal energy are the other factors which stabilize the ligand - protein interaction in the present docking studies, wherein the values of electrostatic force of molecules were significant, which is a sign of a good protein-ligand interaction. Presence of polar residues in the substrate binding site reflects a stable electrostatic interaction (Table 5). Presence of multiple H-bonds between the substrate and protein is significant enough for strong bonding interactions [50]. Ligand asparagine interacted well with the protein L-

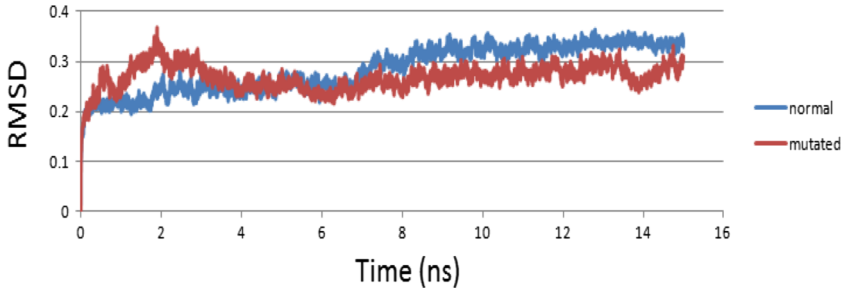

Fig. 14 Plot showing RMSD of normal and mutated L-Asparaginase with bound substrate L- Asparagine for 15 ns

Asparaginase sourced from both *Escherichia coli* and *Pectobacterium carotovorum* in the docking grid.

Native EcA, when docked with L- Asparagine as substrate a binding energy value of −11.86 KCals/mole was obtained which shows good asparaginase activity, whereas for PecA, it was found to be −10.26 KCals/mole. The mutated models show promising binding energy values (−12.01 KCals/mole for EcA and −11.60 KCals/mole for PecA) which clearly say that there is no influence on the catalytic activity of the enzyme even after mutating the immune regions (Table 6). Thus, it was confirmed that protein model with reduced antigenic property without any change in the functionality of the enzyme was obtained.

Molecular Dynamics Simulation Studies

As some of the atoms in the PDB file were missing, they were added with the help of pymol software. The protein is solvated with water molecules in a cubic lattice. 5 Cl^- ions are added to neutralize the system and thereafter energy minimization is carried out to obtain a protein without any steric clashes or inappropriate geometry. Then, simulations were carried out for 15 ns, where an RMSD of 0.25–0.3 nm is observed and the system was found to be stabilized after 8 ns. A graph between the mutated protein with respect to immunogenicity and native protein was drawn where two lines were found to be stabilized, which clearly states that the enzyme variants remained stable even after mutating the residues that are distributed throughout the protein [Fig. 14].

Conclusion

L-Asparaginase, one of the most successful therapeutic which play a vital role in the treatment of acute lymphoblastic leukaemia in children. Even though various organisms are reported to be great producers of enzyme, they have been reported to have the problems of hypersensitivity. So, in the present study, we made an attempt to reduce the immunogenicity by *in silico* engineering. Peptides of B-cell and T-cell epitopes were identified and were mutated at the epitopic sites which has given a 50 % reduction in the immunogenicity score. The models were generated and docked with substrate L- Asparagine to analyze their clinical efficacy and found that they are equally potent in their catalytic function. Further the models were subjected to molecular dynamics simulation studies and found that the models are stable with respect to their structure and activity. Obtained *in silico* results shall pave a way for further enzyme engineering and clinical studies to obtain a more potent L- Asparaginase with reduced event free survival in ALL.

References

1. De Duve C (1966) The significance of lysosome in pathology and medicine. Proc Inst Med Chic 26:73–76
2. Offman MN, Krol M, Patel N, Krishnan S, Liu JZ, Saha V, Bates PA (2011) Rational engineering of L- asparaginase reveals importance of dual activity for cancer cell toxicity. Blood 117:1614–1621
3. Wai Kin Chan, Philip L. Lorenzi, Andriy Anishkin, Preeti Purwaha, David M. Rogers, Sergei, Sukharev, Susan B. Rempe, and John N. Weinstein. (2014). The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. Blood. doi: 10.1182/blood-2013-10-535112.
4. Stams WAG, den Boer ML, Holleman A, Appel IM, Beverloo B, van Wering ER, Janka-Schaub GE, Evans WE, Pieters R (2005) Asparagine synthetase expression is linked with l-asparaginase resistance in *TEL-AML1*-negative but not *TEL-AML1*-positive pediatric acute lymphoblastic leukemia. Blood 105:4223–4225
5. Narta UK, Kanwar SS, Azmi W (2007) Pharmacological and clinical evaluation of L-asparaginase in the treatment if leukaemia. Crit Rev Oncol/Hematol 61:208–221
6. Pieters R, Hunger S, Boos J et al (2011) L-asparaginase treatment in acute lymphoblastic leukemia. Cancer 117:238–249
7. Avramis VI, Sencer S, Periclou AP et al (2002) A randomized comparison of native escherichia coli asparaginase and polyethylene glycol conjugated asparaginase for treatment of children with newly diagnosed standard-risk acute lymphoblastic leukemia: a children's cancer group study. Blood 99:1986–1994
8. Zalewska-Szewczyk B, Gach A, Wyka K et al (2009) The cross-reactivity of antiasparaginase antibodies against different L-asparaginase preparations. Clin Exp Med 9:113–116
9. Raetz EA, Salzer WL (2010) Tolerability and efficacy of L-asparaginase therapy in pediatric patients with acute lymphoblastic leukemia. J Pediatr Oncol 32:554–563
10. Rizzari C, Citterio M, Zucchetti M et al (2006) A pharmacological study on pegylated asparaginase used in front-line treatment of children with acute lymphoblastic leukemia. Haematologica 91:24–31
11. Abuchowski A, Van Es T, Palczuk NC (1979) Treatment of L5178Y tumor bearing BDf mice with a non-immunogenic l-glutaminaseasparaginase. Cancer Treat Rep 63:1127–1129

12. Hawkins DS, Park JR, Thomson BG et al (2004) Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. Clin Cancer Res 10:5335–5341
13. Albertsen BK, Schrøder H, Jakobsen P et al (2002) Antibody formation during intravenous and intramuscular therapy with *Erwinia* asparaginase. Med Pediatr Oncol 38:310–316
14. Klug Albertsen B, Schmiegelow K, Schrøder H et al (2002) Anti-Erwinia asparaginase antibodies during treatment of childhood acute lymphoblastic leukemia and their relationship to outcome: a case–control study. Cancer Chemother Pharmacol 50:117–120
15. Vrooman LM, Supko JG, Neuberg DS et al (2010) Erwinia asparaginase after allergy to *E. coli* asparaginase in children with acute lymphoblastic leukemia. Pediatr Blood Cancer 54:199–205
16. Kumar GT, Prakash NJ, Laxmi N, Krishna TD (2011) Biotechnology based durg delivery by PEGylation method. IJRAP 2(1):95–102
17. Kumar S, Pakshirajan K, Venkata Dasu V (2009) Development of medium for enhanced production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. Appl Microbiol Biotechnol 84:477–486
18. Kumar S, Pakshirajan K, Venkata Dasu V (2010) Assessment of physical process conditions for enhanced production of novel glutaminase-free L-Asparaginase from *Pectobacterium carotovorum* MTCC 1428. Appl Biochem Biotechnol 163:327–337
19. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2001) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739
20. Bailey T L, Elkan C (1994) "Fitting a mixture model by expectation maximization to discover motifs in biopolymers," in Proceedings of the 2nd International Conference on Intelligent Systems for Molecular Biology. AAAI Press, pp 28–36
21. Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer ELL, Eddy SR, Bateman A, Finn RD (2012) Nucleic acids research. Database Issue 40:D290–D301
22. Zhang Q, Wang P, Kim Y (2008) Immune epitope database analysis resource (IEDB-AR). Nucleic Acids Res 36(Web Server issue): W513–W518
23. Emini EA, Hughes JV, Perlow DS, Boger J (1985) Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. J Virol 55:836–839, PMID: 2991600
24. Parker JM, Guo D, Hodges RS (1986) New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with immunogenicity and X-ray-derived accessible sites. Biochemistry 25:5425–5432, PMID: 2430611
25. Nielsen M, Lundegaard C, Lund O (2007) Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. BMC Bioinf 8:238
26. Cantor JR, Yoo TH, Dixit A, Iverson BL, Forsthuber TG, Georgiou G (2011) Therapeutic enzyme deimmunization by combinatorial T-cell epitope removal using neutral drift. PNAS 108(4):1272–1277
27. Hete'nyi C, Spoel VD (2002) Protein Sci 11:1729 [PMID:12070326]
28. Sousa SF, Fernandes PA, Ramos MJ et al (2006) Proteins 65:15, PMID: 16862531
29. Huey R, Morris GM, Olson AJ, Goodsell DS (2007) A semi empirical free energy force field with charge-based desolvation. J Comput Chem 28:145–152
30. Wang B, Relling MV, Storm MC et al (2003) Evaluation of immunologic crossreaction of antiasparaginase antibodies in acute lymphoblastic leukemia (ALL) and lymphoma patients. Leukemia 17:1583–1588
31. Berendsen HJC, van der Spoel D, van Drunen R (1995) GROMACS: a message-passing parallel molecular dynamics implementation. Comput Phys Commun 91:43–56
32. Erik L, Berk H, van der Spoel D (2001) GROMACS 3.0: a package for molecular simulation and trajectory analysis. J Mol Model 7:306–317
33. Van der Spoel D, Lindahl E, Hess B, van Buuren AR, Apol E, Meulenhoff PJ, Tieleman DP, Sijbers ALTM, Feenstra KA, van Drunen R, Berendsen HJC (2005) Gromacs User Manual version 4.0. www.gromacs.org
34. Schüttelkopf AW, van Aalten DMF (2004) PRODRG - a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallogr D60:1355–1363
35. Kaushik Ramakrishanan S, Krishna V, Kumar SV, Lakshmi BS, Anishetty S, Gautham P (2008) Molecular dynamics simulation of lipases. Int J Integr Biol 2:204–213
36. Clementi A (1922) Desamidation enzymatique de l' L-Asparagine. Archives Internationales de Physiologie 19:369
37. Ramya LN, Mukesh D, Rekha VPB, Pulicherla KK (2012) L-Asparaginase as potent anti-leukemic agent and its significance of having reduced glutaminase side activity for the better treatment of acute lymphoblastic leukaemia. Appl Biochem Biotechnol 167(8):2144–2159
38. Kidd JG (1953) Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum—course of transplanted cancers of various kinds in mice and rats given guinea pig serum or rabbit serum. J Exp Med 98:565–582
39. Wade HE, Elsworth R, Herbert D (1968) A new L-asparaginase with anti-tumor activity. Lancet 2:776–777
40. Manna S, Sinha A, Sadhukhan R, Chakrabarty SL (1995) Purification, characterization and antitumor activity of L-asparaginase isolated from Pseudomonas stutzeri MB-405. Curr Microbiol 30:291–298
41. Bander NH et al (2005) Phase I trial of 177lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer. J Clin Oncol 23: 4591–4601
42. Holgate RG, Baker MP (2009) Circumventing immunogenicity in the development of therapeutic antibodies. IDrugs 12:233–237
43. Macfarlane DJ et al (2006) Safety, pharmacokinetic and dosimetry evaluation of the proposed thrombus imaging agent 99mTc-DI-DD-3B6/22-80B3 Fab 0. Eur J Nucl Med Mol Imaging 33:648–656
44. Wang P, Sidney J, Dow C, Mothé B, Sette A, Peters B (2008) A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLoS Comput Biol 4(4): e1000048
45. Wang P et al (2008) A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLoS Comput Biol 4:e1000048
46. Moola ZB, Scawen MD, Atkinson T, Nicholls DJ (1994) Erwinia chrysanthemi L-asparaginase: epitope mapping and production of antigenically modified enzymes. Biochem J 302:921–927
47. Jianhua C, Yujun W, Ruibo J, Min W, Wutong W (2006) Probing the antigenicity of E. coli L-asparaginase by mutational analysis. Mol Biotechnol 33(1):57–65
48. Ramya LN, Doble M, Rekha VPB, Pulicherla KK (2011) In silico engineering of L-Asparaginase to have reduced glutaminase side activity for effective treatment of acute lymphoblastic leukaemia. J Pediatr Hematol Oncol 33(8):617–621
49. Derst C, Henseling J, Rohm KH (1992) Probing the role of threonine and serine residues of E.coli asparaginase II by site-specific mutagenesis. Protein Eng 5:785–789, PubMed: 1287659
50. Patil R, Das S, Stanley A et al (2010) Optimized hydrophobic interactions and hydrogen bonding at the target-ligand interface leads the pathways of drug-designing. PLoS ONE 5(8):1–10