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Rhizobium etli asparaginase II

An alternative for acute lymphoblastic leukemia (ALL) treatment

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Bacterial L-asparaginase has been a universal component of therapies for childhood acute lymphoblastic leukemia since the 1970s. Two principal enzymes derived from *Escherichia coli* and *Erwinia chrysanthemi* are the only options clinically approved to date. We recently reported a study of recombinant L-asparaginase (AnsA) from *Rhizobium etli* and described an increasing type of AnsA family members. Sequence analysis revealed four conserved motifs with notable differences with respect to the conserved regions of amino acid sequences of type I and type II L-asparaginases, particularly in comparison with therapeutic enzymes from *E. coli* and *E. chrysanthemi*. These differences suggested a distinct immunological specificity. Here, we report an in silico analysis that revealed immunogenic determinants of AnsA. Also, we used an extensive approach to compare the crystal structures of *E. coli* and *E. chrysantemi* asparaginases with a computational model of AnsA and identified immunogenic epitopes. A three-dimensional model of AsnA revealed, as expected based on sequence dissimilarities, completely different folding and different immunogenic epitopes. This approach could be very useful in transcending the problem of immunogenicity in two major ways: by chemical modifications of epitopes to reduce drug immunogenicity, and by site-directed mutagenesis of amino acid residues to diminish immunogenicity without reduction of enzymatic activity.

Keywords: ALL, asparaginase II, immunogenic epitopes, immuno-Logo, *Rhizobium etli*

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Introduction

The last two decades have been characterized by the emergence of a great number of proteins as potential drug candidates to treat diseases in patients with various cancers, or as treatments for heart attacks, strokes, cystic fibrosis, Gaucher disease, diabetes, anemia, hemophilia and inflammation. These therapeutic proteins are extracted from plants, microbes or human cells or are engineered in the laboratory for pharmaceutical use.[1] The majority of them are manufactured using bacterial systems and nonhuman mammalian cell lines and recombinant techniques,[2] and they constitute an important class of therapeutics used to replace patients' deficiencies in critical blood-borne growth factors and to strengthen the immune system to fight cancer and infectious diseases.[3] Twenty-nine years have passed since approval of recombinant human insulin by the FDA, and a remarkable expansion has been seen in the number of therapeutic applications of proteins.[4,5] Today we are witnessing a continuous rise in the number of approved protein therapeutics,[6] and there is little doubt that biopharmaceuticals have the potential to become the medicines of the future. To date, more than 130 proteins (over 95 of which are recombinant) are currently approved for use by the FDA, and many more are in development.[7,8] Recombinant DNA technology not only allows therapeutic proteins to be produced on a large scale but also, by using the same methodology, protein molecules

can be engineered and improved.[9] The genetic modifications introduced into a protein have many advantages over chemical modifications, since engineered entities must be considered biocompatible and biodegradable. In conjunction, changes introduced into a molecule avoid rare errors in gene transcription or translation, and protein preparations do not contain residual amounts of harsh chemicals used in the purification process.[10]

From a therapeutic perspective, proteins offer the distinct advantage of specific mechanisms of action and high potency. Despite these advantages, biotech products must overcome the hurdles posed by their high molecular weights, short half-lives, instability and immunogenicity. Several strategies have been evaluated in an effort to improve the current limitations of therapeutic peptides and proteins in the creation of so-called "second-generation" protein therapeutics. Most efforts are centered on two approaches: either a change in the agent itself (e.g., mutations in protein structure or covalent attachment of moieties)[11] or by a change in formulation.[12] In contrast to modifying the protein structure, covalent chemical attachment of compounds, such as polyethylene glycol (PEG) or polysialic acid (PSA), to a therapeutic protein represents a relatively new approach. Drug formulation systems, such as liposomes, polymeric microspheres and polymeric nanoparticles, are another means to help overcome the current limitations of protein therapeutics.[13] A third strategy to minimize immunogenicity of therapeutic proteins is related to identification of immunogenic epitopes on the protein by using in silico prediction tools and by bioengineering less immunogenic proteins.[1,14]

Immunogenicity of Therapeutic Proteins

Therapeutic proteins can be classified by their function or application into four main groups: (1) proteins with enzymatic or regulatory activity, (2) proteins with special targeting activity, (3) protein vaccines and (4) Proteins used for disease diagnosis.[15]

Protein therapeutics have several advantages over small-molecule drugs: proteins are highly specific and carry on a complex set of functions that cannot be mimicked by simple chemical compounds; all of them are highly specific with less potential to interfere with normal biological processes; often they are well tolerated and are less likely to elicit an immune response; they can provide effective replacement treatment options without the need for gene therapy; the approval times for therapeutic proteins may be faster than those for small-molecule drugs; and finally, proteins are unique in form and are able to obtain far-reaching patent protections.[7,16]

The attractiveness of therapeutic proteins in fact is related to the high specificity by which they execute diverse functions.[17] The introduction of recombinant human proteins, such as recombinant human erythropoietin, insulin proteins, growth hormones and cytokines, has revolutionized the treatment of many diseases.[18-20] It is currently estimated that there are 25,000 to 40,000 different genes in the human genome, which suggests that many more therapeutic proteins will soon become available. However, administration of therapeutic proteins in multiple doses over extended periods has been shown to induce immune responses. These immune responses can be as minor as local irritation or as serious as cardiovascular collapse.[21,22] Most biotechnologically derived proteins induce an unwanted immune response that is triggered by more than a single factor. The immunological response is complex and, in addition to antibody formation, other events, such as T-cell activation or innate immune response activation, can contribute to potential adverse responses.[23] The consequences of an immune reaction range from the transient appearance of antibodies without any clinical significance to severe life-threatening conditions.[24] Additional factors that may influence the immunogenicity of therapeutic proteins are attributable to the patient, the disease, or components of products. Factors in patients include underlying disease, genetic background, immune status (including immunomodulating therapy) and dosing schedule. Components and production steps also influence the likelihood of an immune response, based on the manufacturing process, the formulation and stability characteristics. In the case of a recombinant protein, many factors can contribute to alterations in a protein's ability to elicit an antibody response, including glycosylation, contaminants, temperature changes and storage media.[25]

L-Asparaginase: A Potent Antitumor Enzyme

A major potential application of therapeutic enzymes is in cancer treatment. These enzymes fall into two categories of therapeutic proteins: agents that degrade the small molecules for which neoplastic tissues have a requirement, and agents that degrade macromolecules. Important features that distinguish enzyme drugs from other types of drugs include enzymes often binding and acting on their targets with great affinity and specificity and enzymes being catalytic and able to convert multiple molecules to the desired product(s).[26]

Acute lymphoblastic leukemia (ALL) involves the malignant transformation of a clone of cells from the bone marrow, where early lymphoid precursors proliferate and can replace normal cells. For several years, childhood ALL treatment included the use of asparaginases, since lymphoma cells cannot synthesize L-asparagine (L-Asn) and thus depend on external uptake of this amino acid for growth.[27,28] Asparaginase has proved promising for the treatment of ALL, considering that tumor cells are deficient in aspartate-ammonia ligase activity, which restricts their ability to synthesize the normally nonessential amino acid L-asparagine. The action of asparaginase does not affect the functioning of normal cells, which are able to synthesize enough for their own requirements, but it reduces the free exogenous L-Asn concentration and so it induces a state of fatal starvation in susceptible tumor cells. These bacterial-type asparaginases have shown a beneficial pharmacological effect in the treatment of ALL by depleting blood L-Asn pools.[29-33] Nowadays, L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* are used in the treatment of ALL.[34] Their therapeutic efficacy is well established. However, in some patients their beneficial effect rarely occurs without some evidence of toxicity, which in part is due to the glutaminase activity

of these enzymes[35,36] and also, primarily, because both native and modified PEG-asparaginase versions of the enzyme are immunogenic, leading to adverse immune responses to both versions.[37] With these factors in mind, L-asparaginases with high specificity for L-Asn and negligible activity against L-glutamine are indicated for anticancer therapy.[38-40] Several asparaginases have been investigated in order to characterize enzymes with less toxic side effects.[41-44]

Recently, we reported that L-asparaginase from *Rhizobium etli* (AnsA) hydrolyzes L-Asn at similar levels as the L-asparaginase from *E. chrysanthemi*.[45] With regard to substrate specificity, enzymatic activities were not detected for aspartate, D-asparagine or glutamine as substrates. The activity of AnsA was specific for L-Asn. We also confirmed that AnsA does not show glutaminase activity. In addition, we described 350 proteins of the rhizobial-type asparaginases grouped in this subfamily, wherein the majority are derived from microorganisms that inhabit soil or marine environments. Sequence analysis revealed four conserved motifs [RSx(2)KPxQA; ALxCASH; NCSGKHxGxL; and AMx(3)Px(2) VAGxGrx(2)TxLM], with notable differences with respect to the conserved regions of amino acid sequences of type I and type II l-asparaginases, particularly on comparison with therapeutic enzymes of *E. coli* and *E. chrysanthemi*. These differences suggest a distinct immunological specificity. In summary, considering the biochemical properties of L-asparaginase of *R. etli*, e.g., the glutaminase-free asparaginase activity, and its different amino acid sequence compared with *E. coli* and *E. chrysantemi* asparaginases, led us to propose it as a potential therapeutic enzyme for ALL treatment.[46]

In Silico Immunogenicity Profile of Rhizobial L-asparaginase (AnsA)

In order to determine the immunogenic determinants of asparaginases, we used an in silico approach to compare amino acid sequences and immunogenic profiles of the three enzymes. The first question we asked was if *E. coli* and *E. chrysantemi* asparaginases are phylogenetically and immunogenically closely related. To this end, sequence comparison among asparaginases was performed by BLAST analysis (http://blast.ncbi.nlm.nih.gov). *E. coli* and *E. chrysantemi* asparaginases were closely related (47% identity; **Fig. 1A**), whereas AnsA showed completely different sequence (6% identity) from the other two asparaginases. A subsequent analysis of the amino acid sequences of the three enzymes was performed to investigate antigenic determinants (epitopes) by using the servers BCPREDS (B-cell epitope prediction server; http://ailab.cs.iastate.edu/bcpreds/)[47,48] and Epitopia (http://epitopia.tau.ac.il/). The antigenic determinants are shown in **Table 1**.

Table 1. BCPreds prediction of B-cell epitopes from asparaginases

Epitope sequences of L-asparaginases		
Amino acid position	**BCPred epitope sequence**	**BCPred score**
***Rhizobium etli* L-asparaginase**		
186	TDG CNL PTP AFP	0.988
150	GAG TDG YHL PDH	0.970
338	GVT TGG VSF PFK	0.929
35	YAL GNP TRM TLA	0.813
***Escherichia coli* L-asparaginase**		
7	LAT GGT IAG GGD	1
104	KCD KPV VMV GAM	0.99
242	AGV GNG NLY KSV	0.984
73	INT DCD KTD GFV	0.945
159	DVT KTN TTD VAT	0.898
58	SQD MND NVW LTL	0.891
122	SAD GPF NLY NAV	0.81
***Erwinia chrysantemi* L-asparaginase**		
312	TRT SDP KVI QEY	0.991
108	TVK SDK PVV FVA	0.983
277	TRT GNG IVP PDE	0.972
21	ATG TQT TGY KAG	0.958
244	GIV YAG MGA GSV	0.934
264	RKA LEK GVV VMR	0.898
223	LYG YQD DPE YLY	0.883
8	VIL ATG GTI AGS	0.802

Epitope length: 12 amino acids, and specificity at 75%. Only BCPRED score values ≥ 0.8 were considered.

The immunogenic epitopes with higher values were mapped in the *E. coli* and *E. chrysantemi* asparaginase protein sequences (**Fig. 1A**). Shaded residues represent the most highly immunogenic determinants predicted by Epitopia analysis, whereas underlined epitopes show BCPREDS predictions. As expected, *E. coli* and *E. chrysantemi* asparaginases shared immunogenic residues (**Fig. 1A**, blue and green), and at least five of them were predicted by two servers (**Fig. 1**, green). In order to evaluate the conservation of immunogenic regions in AsnA and its relatives, a BLAST search was run using AsnA of *R. etli* as seed, with an

Figure 1 (See opposite page). (**A**) Immunogenic profiles of the *E. coli* and *E. chrysantemi* asparaginases. Highly immunogenic residues identified by using Epitopia are shown (shaded residues). Red, no shared epitopes; blue, shared epitopes. Immunogenic epitopes identified by using BCPREDS are underlined. Shared epitopes predicted by BCPREDS are shown in green. (**B**) Immunogenic profiles of AnsA. Residues in red had the highest Epitopia scores. BCPREDS epitopes are indicated by underlining. Four conserved motifs from rhizobial-type asparaginases are shown in boldface letters. Cyan, score = 0.7 to 0.79; green, score ≥ 8. Shaded residues show antigenic motifs from rhizobial-type asparaginases, identified using Immuno-Logo.

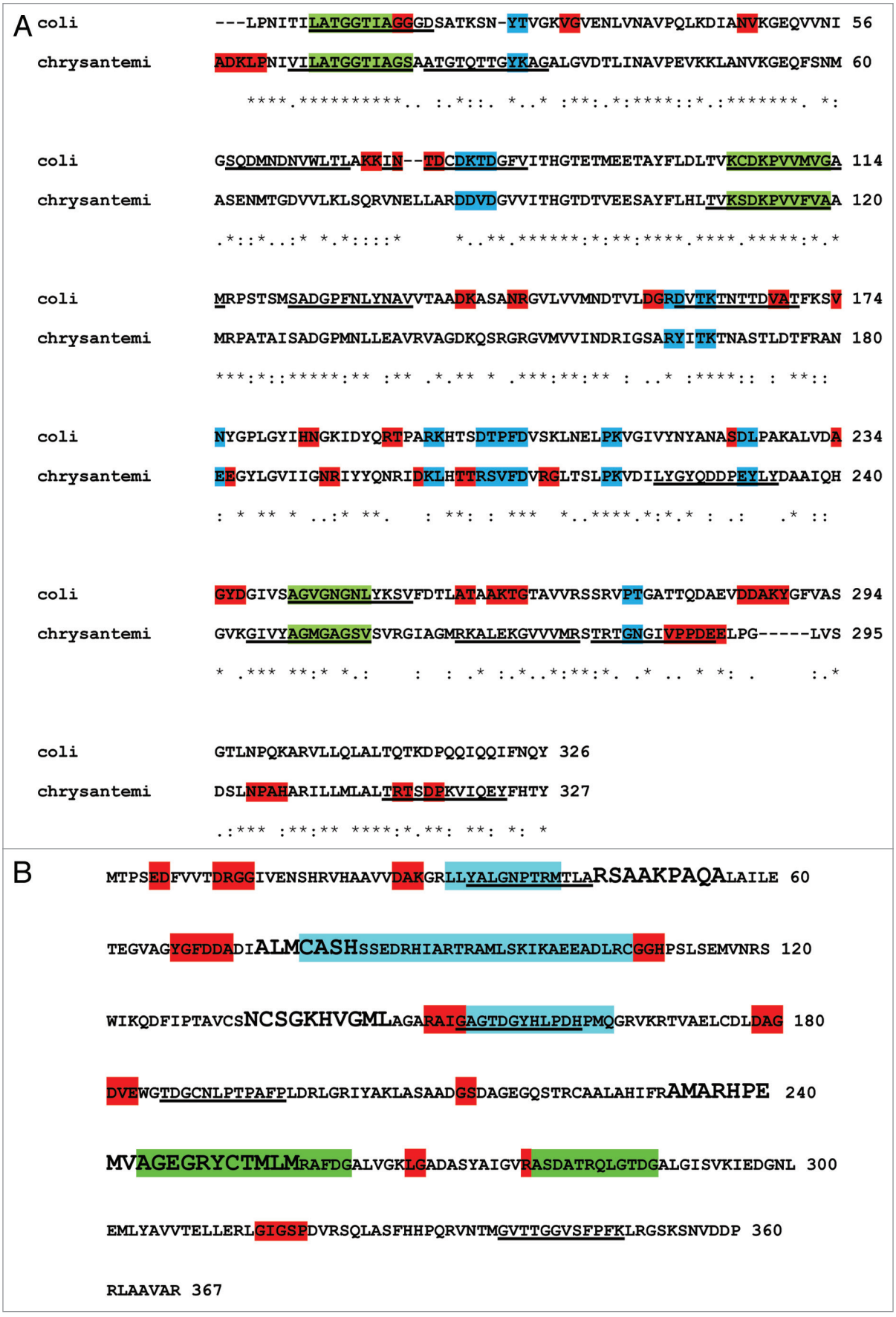

Figure 1. For figure legend, see page 32.

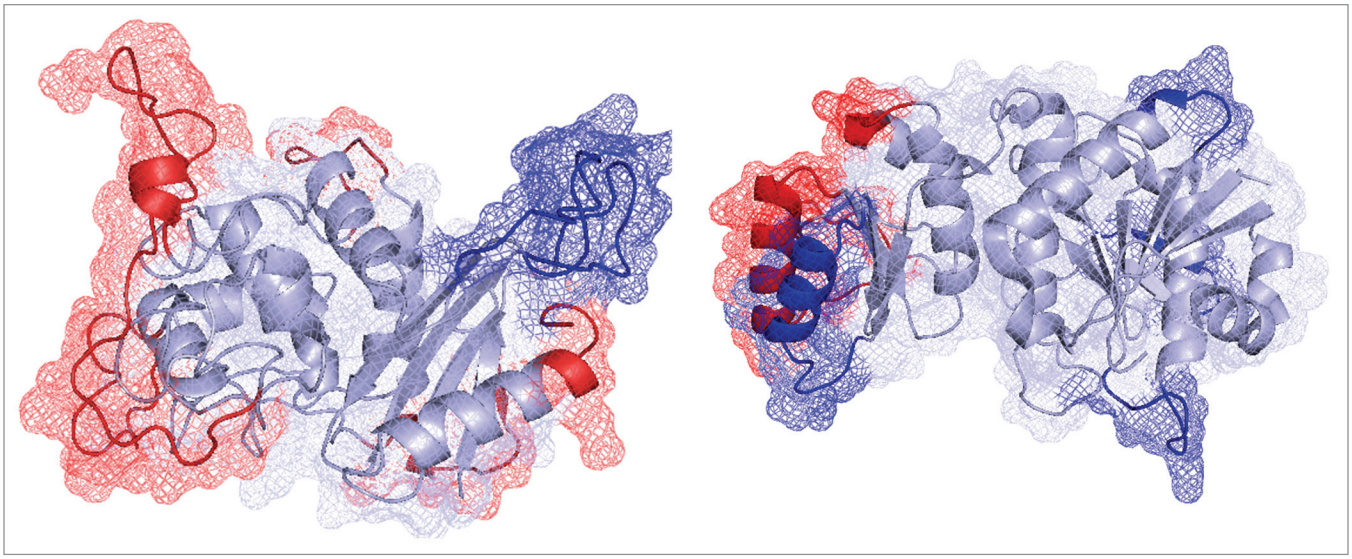

Figure 2. Computational model of the *R. etli* AsnA (left) and the crystal structure of the *E. chrysantemi* asparaginase (right). Immunogenic regions with a score of ≥ 0.8 are shown in blue. In red are immunogenic regions with scores between 0.7 and 0.799. Both proteins exhibited a different fold and different immunogenic determinants.

E-value ≤ 10^{-5}, against the NR database. A total of 372 protein sequences were retrieved from this sequence comparison. These sequences were filtered at 90% identity to exclude overrepresentation biases by using the CD-hit program,[49] leaving a set of 284 proteins. These sequences were then aligned by using the CLUSTAL program[50] with default parameters, and their corresponding immunogenic regions were located by Epitopia analysis (ImmunoLogo; Fig. S1). From this comparison we found three motifs with high immunogenicity values (score, > 0.8) (**Fig. 1B**, cyan) and two with a score between 0.7 and 0.8 (**Fig. 1B**, green). We also found that two rhizobial-type family conserved motifs previously identified overlap with the immunogenic regions, suggesting that AnsA immunogenic properties may be a general property for AsnA homologs of *R. etli* (**Fig. 1B**).

Structural Comparison of *E. coli*, *E. chrysantemi*, and *R. etli* Asparaginases

The crystal structures of *E. coli* asparaginase (PDB entry 1JJA) and *E. chrysantemi* (PDB entry 1HG1) were analyzed. These structures were compared using the structural alignment Topmatch server (http://topmatch.services.came.sbg.ac.at). Additionally, a computational model of the three-dimensional structure of AsnA was built by using the CPHmodels 3.2 server (http://www.cbs.dtu.dk/services/CPHmodels) with the structure of OXA10, a class d β-lactamase from *Pseudomonas aeruginosa* (PDB entry 1E3U.D), as a template. In order to refine the model, it was minimized using the Gromacs server (http://lorentz.immstr.pasteur.fr/gromacs). Finally, the RAMPAGE program (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) was used to validate the stereochemical quality of the resulting three-dimensional model. After analyzing the Ramachandran plot, 88.5% of the residues were in favored and allowed regions, and only 7.6% were in disallowed regions. All the bond distances, angles, and dihedrals fulfilled the normal limits for polypeptide chains. The model includes 314 of the 371 residues of AsnA. The three-dimensional model of *R. etli* asparaginase was then compared with crystal structures of the *E. coli* and *E. chrysantemy* asparaginases (**Fig. 2**). Structurally, AsnA has a strong similarity to proteins belonging to the β-lactamase/transpeptidase-like fold. In contrast, asparaginases from *E. coli* and *E. chrysantemi* belong to the glutaminase/asparaginase fold, suggesting different evolutionary origins or a large divergence in terms of sequence and structure. In order to corroborate this finding, we performed a structural superposition of AsnA of the three organisms, with similar results, reinforcing the notion that they share different structural determinants, i.e., the superposition RMSD values in all α carbons showed high structural similarities between *E. coli* and *E. chrysantemi* asparaginases (RMSD = 1.68 Å), whereas both proteins were dissimilar to AsnA (RMSD = 21.86 Å). High RMSD values indicate a lack of structural correspondence. In order to identify continuous epitopes in the proteins, we used the ElliPro server. In brief, this method identifies potential epitopes protruding from the protein's globular surface and, together with a residue clustering algorithm, allows the prediction of antibody epitopes for a given protein.[51] The comparison of crystal structures of *E. coli* and *E. chrysantemi* asparaginases regarding the three-dimensional model of AsnA revealed, as expected based on sequence dissimilarities, a completely different folding and different immunogenic epitopes.

Conclusions

The computational prediction of therapeutic protein epitopes is of important theoretical and practical value, as experimental identification of such epitopes is costly and time-consuming. This approach could be useful in reducing the problem of immunogenicity with the use of therapeutic proteins. First, we can identify

those immunogenic residues included in conserved motifs that are presumably involved in enzymatic activity or protein stability. Identification of such residues will allow us to change by site-directed mutagenesis the nonconserved residues to reduce epitope immunogenicity without loss of enzymatic activity or stability. Second, the conserved residues with high immunogenicity could be subjected to covalent chemical attachment of compounds, such as PEG or PSA, to reduce immunogenicity. On the other hand, evaluation of conserved immunogenic regions is a strong approach to evaluate whether any given therapeutic protein is a suitable candidate to be included in a disease treatment or if its immunogenic profile warns us about its use as an alternative for clinical treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:

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References

1. Adair F, Ozanne D. The immunogenicity of therapeutic proteins. Biopharm International 2002; 15:30-6.
2. Matasci M, Hacker DL, Baldi L, Wurm FM. Recombinant therapeutic protein production in cultivated mammalian cells: current status and future prospects. Drug Discov Today 2008; 5:e37-42; http://dx.doi.org/10.1016/j.ddtec.2008.12.003.
3. Nayak KA. Advances in therapeutic protein production and delivery. Int J Pharmacy and Pharma Sci 2010; 2:1-5.
4. Johnson IS. Human insulin from recombinant DNA technology. Science 1983; 219:632-7; PMID:6337396; http://dx.doi.org/10.1126/science.6337396.
5. Purcell RT, Lockey RF. Immunologic responses to therapeutic biologic agents. J Investig Allergol Clin Immunol 2008; 18:335-42; PMID:18973096.
6. Walsh G. Biopharmaceutical benchmarks 2006. Nat Biotechnol 2006; 24:769-76; PMID:16841057; http://dx.doi.org/10.1038/nbt0706-769.
7. Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. Nat Rev Drug Discov 2008; 7:21-39; PMID:18097458; http://dx.doi.org/10.1038/nrd2399.
8. Baker MP, Reynolds HM, Lumicisi B, Bryson CJ. Immunogenicity of protein therapeutics: The key causes, consequences and challenges. Self Nonself 2010; 1:314-22; PMID:21487506.
9. Tomlinson IM. Next-generation protein drugs. Nat Biotechnol 2004; 22:521-2; PMID:15122287; http://dx.doi.org/10.1038/nbt0504-521.
10. Kamionka M. Engineering of therapeutic proteins production in *Escherichia coli*. Curr Pharm Biotechnol 2011; 12:268-74; PMID:21050165; http://dx.doi.org/10.2174/138920111794295693.
11. Jenkins N. Modifications of therapeutic proteins: challenges and prospects. Cytotechnology 2007; 53:121-5; PMID:19003198; http://dx.doi.org/10.1007/s10616-007-9075-2.
12. Soderquist RG, Milligan ED, Sloane EM, Harrison JA, Douvas KK, Potter JM, et al. PEGylation of brain-derived neurotrophic factor for preserved biological activity and enhanced spinal cord distribution. J Biomed Mater Res A 2009; 91:719-29; PMID:19048635; http://dx.doi.org/10.1002/jbm.a.32254.
13. Banakar UV. Advances and opportunities in delivery of therapeutic proteins and peptides. J Biomater Appl 1997; 11:377-429; PMID:9178093.
14. De Groot AS, Martin W. Reducing risk, improving outcomes: bioengineering less immunogenic protein therapeutics. Clin Immunol 2009; 131:189-201; PMID:19269256; http://dx.doi.org/10.1016/j.clim.2009.01.009.
15. Sutradhar KB, Khatun S, Al Mamun A, Begum M. Distribution and elimination of protein therapeutics: a review. S J Pharm Sci 2011; 4:1-12.
16. Carter PJ. Introduction to current and future protein therapeutics: a protein engineering perspective. Exp Cell Res 2011; 317:1261-9; PMID:21371474; http://dx.doi.org/10.1016/j.yexcr.2011.02.013.
17. Wright CM, Wright RC, Eshleman JR, Ostermeier M. A protein therapeutic modality founded on molecular regulation. Proc Natl Acad Sci U S A 2011; 108:16206-11; PMID:21930952; http://dx.doi.org/10.1073/pnas.1102803108.
18. Oberg K, Alm G, Magnusson A, Lundqvist G, Theodorsson E, Wide L, et al. Treatment of malignant carcinoid tumors with recombinant interferon alfa-2b: development of neutralizing interferon antibodies and possible loss of antitumor activity. J Natl Cancer Inst 1989; 81:531-5; PMID:2466128; http://dx.doi.org/10.1093/jnci/81.7.531.
19. Gelfand EW. Antibody-directed therapy: past, present, and future. J Allergy Clin Immunol 2001; 108(Suppl):S111-6; PMID:11586277; http://dx.doi.org/10.1067/mai.2001.117824.
20. Olsen E, Duvic M, Frankel A, Kim Y, Martin A, Vonderheid E, et al. Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. J Clin Oncol 2001; 19:376-88; PMID:11208829.
21. Gruchalla R. Understanding drug allergies. J Allergy Clin Immunol 2000; 105:S637-44; PMID:10856171; http://dx.doi.org/10.1067/mai.2000.106156.
22. Schellekens H. Factors influencing the immunogenicity of therapeutic proteins. Nephrol Dial Transplant 2005; 20(Suppl 6):vi3-9; PMID:15958824; http://dx.doi.org/10.1093/ndt/gfh1092.
23. Cantor JR, Yoo TH, Dixit A, Iverson BL, Forsthuber TG, Georgiou G. Therapeutic enzyme deimmunization by combinatorial T-cell epitope removal using neutral drift. Proc Natl Acad Sci U S A 2011; 108:1272-7; PMID:21209329; http://dx.doi.org/10.1073/pnas.1014739108.
24. Chirino AJ, Ary ML, Marshall SA. Minimizing the immunogenicity of protein therapeutics. Drug Discov Today 2004; 9:82-90; PMID:15012932; http://dx.doi.org/10.1016/S1359-6446(03)02953-2.
25. Sharma B. Immunogenicity of therapeutic proteins. Part 2: impact of container closures. Biotechnol Adv 2007; 25:318-24; PMID:17337336; http://dx.doi.org/10.1016/j.biotechadv.2007.01.006.
26. Sabu A. Sources, properties and applications of microbial therapeutic enzymes. Int J Biotechnol 2003; 2:334-41.
27. Cooney DA, Handschumacher RE. L-asparaginase and L-asparagine metabolism. Annu Rev Pharmacol 1970; 10:421-40; PMID:4911021; http://dx.doi.org/10.1146/annurev.pa.10.040170.002225.
28. Cantor JR, Panayiotou V, Agnello G, Georgiou G, Stone EM. Engineering reduced-immunogenicity enzymes for amino acid depletion therapy in cancer. Methods Enzymol 2012; 502:291-319; PMID:22208990; http://dx.doi.org/10.1016/B978-0-12-416039-2.00015-X.
29. Boyse EA, Old LJ, Campbell HA, Mashburn LT. Suppression of murine leukemias by L-asparaginase. Incidence of sensitivity among leukemias of various types: comparative inhibitory activities of guinea pig serum L-asparaginase and Escherichia coli L-asparaginase. J Exp Med 1967; 125:17-31; PMID:5334543; http://dx.doi.org/10.1084/jem.125.1.17.
30. Campbell HA, Mashburn LT, Boyse EA, Old LJ. Two L-asparaginases from *Escherichia coli* B. Their separation, purification, and antitumor activity. Biochemistry 1967; 6:721-30; PMID:5337885; http://dx.doi.org/10.1021/bi00855a011.
31. Gallagher MP, Marshall RD, Wilson R. Asparaginase as a drug for treatment of acute lymphoblastic leukaemia. Essays Biochem 1989; 24:1-40; PMID:2676522.
32. Mashburn LT, Wriston JC Jr. Tumor Inhibitory effect of L-asparaginase from *Escherichia coli*. Arch Biochem Biophys 1964; 105:450-2; PMID:14186753; http://dx.doi.org/10.1016/0003-9861(64)90032-3.
33. Roberts J, Prager MD, Bachynsky N. The antitumor activity of *Escherichia coli* L-asparaginase. Cancer Res 1966; 26:2213-7; PMID:5331901.
34. Dinndorf PA, Gootenberg J, Cohen MH, Keegan P, Pazdur R. FDA drug approval summary: pegaspargase (oncaspar) for the first-line treatment of children with acute lymphoblastic leukemia (ALL). Oncologist 2007; 12:991-8; PMID:17766659; http://dx.doi.org/10.1634/theoncologist.12-8-991.
35. Ashworth LA, MacLennan AP. Comparison of the L-asparaginases from *Escherichia coli* and *Erwinia carotovora* as immunosuppressants. Cancer Res 1974; 34:1353-9; PMID:4596965.
36. Moola ZB, Scawen MD, Atkinson T, Nicholls DJ. *Erwinia chrysanthemi* L-asparaginase: epitope mapping and production of antigenically modified enzymes. Biochem J 1994; 302:921-7; PMID:7945221.
37. Zeidan A, Wang ES, Wetzler M. Pegasparaginase: where do we stand? Expert Opin Biol Ther 2009; 9:111-9; PMID:19063697; http://dx.doi.org/10.1517/14712590802586058.
38. Distasio JA, Niederman RA, Kafkewitz D. Antilymphoma activity of a glutaminase-free L-asparaginase of microbial origin. Proc Soc Exp Biol Med 1977; 155:528-31; PMID:331343.
39. Hawkins DS, Park JR, Thomson BG, Felgenhauer JL, Holcenberg JS, Panosyan EH, et al. Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. Clin Cancer Res 2004; 10:5335-41; PMID:15328169; http://dx.doi.org/10.1158/1078-0432.CCR-04-0222.
40. Kumar S, Venkata Dasu V, Pakshirajan K. Purification and characterization of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. Bioresour Technol 2011; 102:2077-82; PMID:20832300; http://dx.doi.org/10.1016/j.biortech.2010.07.114.

41. Abdel-Fattah YR, Olama ZA. L-Asparaginase production by *Pseudomonas aeruginosa* in solid-state culture: Evaluation and optimization of culture conditions using factorial designs. Process Biochem 2002; 38:115-22; http://dx.doi.org/10.1016/S0032-9592(02)00067-5.
42. Elzainy TA, Ali TH. Detection of the antitumor glutaminase-asparaginase in the filamentous fungi. J Appl Sci 2006; 6:1389-95; http://dx.doi.org/10.3923/jas.2006.1389.1395.
43. Fisher SH, Wray LV Jr. *Bacillus subtilis* 168 contains two differentially regulated genes encoding L-asparaginase. J Bacteriol 2002; 184:2148-54; PMID:11914346; http://dx.doi.org/10.1128/JB.184.8.2148-2154.2002.
44. Mukherjee J, Majumdar S, Scheper T. Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*. Appl Microbiol Biotechnol 2000; 53:180-4; PMID:10709980; http://dx.doi.org/10.1007/s002530050006.
45. Kotzia GA, Labrou NE. L-Asparaginase from *Erwinia Chrysanthemi* 3937: cloning, expression and characterization. J Biotechnol 2007; 127:657-69; PMID:16984804; http://dx.doi.org/10.1016/j.jbiotec.2006.07.037.
46. Moreno-Enriquez A, Evangelista-Martinez Z, González-Mondragon EG, Calderón-Flores A, Arreguín R, Perez-Rueda E, et al. Biochemical characterization of recombinant L-asparaginase (AnsA) from *Rhizobium etli*, a member of an increasing rhizobial-type family of L-asparaginases. J Microbiol Biotechnol 2012; 22:292-300; PMID:22450783; http://dx.doi.org/10.4014/jmb.1107.07047.
47. Saha S, Raghava GPS. BcePred:Prediction of Continuous B-Cell Epitopes in Antigenic Sequences Using Physico-chemical Properties. In Nicosia G, Cutello V, Bentley PJ, Timis J, eds. ICARIS. LNCS 3239. Springer, 2004: 197-204.
48. El-Manzalawy Y, Dobbs D, Honavar V. Predicting linear B-cell epitopes using string kernels. J Mol Recognit 2008; 21:243-55; PMID:18496882; http://dx.doi.org/10.1002/jmr.893.
49. Li W, Jaroszewski L, Godzik A. Clustering of highly homologous sequences to reduce the size of large protein database. Bioinformatics 2001; 17:282-3; PMID:11294794; http://dx.doi.org/10.1093/bioinformatics/17.3.282.
50. Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and 4 ClustalX. Curr Prot Bioinformatics 2002; Chapter 2. Unit 2.3.
51. Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, Sette A, et al. ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics 2008; 9:514; PMID:19055730; http://dx.doi.org/10.1186/1471-2105-9-514.