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Bioinformatic Analysis of L-Asparaginase Structures in Halophilic (*Bacillus subtilis*), Mesophilic (*Kibdelosporangium*), and Thermophilic (*Thermococcus kodakarensis*) Bacteria: New Insights on L-asparaginase as a Potent Antileukemic Agent

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Abstract

Background: The L-asparaginase enzyme is used as an anticancer agent in treating acute lymphoblastic leukemia (ALL). Moreover, it has widespread applications in medicine, food, and pharmaceutical industries.

Methods: The nucleotide and amino acid sequences of L-asparaginase derived from *Bacillus subtilis* BEST7613, *Kibdelosporangium* sp. MJ126-NF4, and *Thermococcus kodakarensis* KOD1 have been obtained from the GenBank and the NCBI databases. The EMBOSS Water pairwise sequence alignments were performed using ClustalW 1.83. Prediction of secondary and tertiary protein structures of the different L-asparaginase molecules studied was done using SWISS-MODEL software. In addition, the protein domains of L-asparaginase originating from the three mentioned bacteria were analyzed using PROSITE software. Theoretical isoelectric point (pI), molecular weight, and amino acid composition were predicted using the protein pI calculator (http://isoelectric.ovh.org/).

Results: Despite the structural differences in L-asparaginase enzymes in the three bacterial strains, there were no differences in their functional characteristics, including molecular weight, pI, and functional domain.

Conclusion: Analyzing structural differences and finding functional similarities can be useful in designing drugs with higher stability and biological half-life. Our analysis showed that proteins with different structures could have similar functional characteristics, which proves the codon usage hypothesis.

Keywords: Asparaginase, Lymphoblastic leukemia, Bioinformatics

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Introduction

Acute lymphoblastic leukemia (ALL) is a group of heterogeneous disorders that arise from the clonal proliferation of malignant lymphoblastic cells in the bone marrow (1). Among chemotherapies for ALL, L-asparaginase extracted from *Escherichia coli* is known as the basic line of treatment. Despite the possible side effects of L-asparaginase, including encephalopathy, thrombosis, and hepatic or pancreatic failure, it has been shown that L-asparaginase can improve survival rate in children (2). L-asparaginase is a tetrameric enzyme found in various animal and plant species and microorganisms. This enzyme can break down L-asparagine into aspartic acid and ammonia (3). L-asparaginase enzyme disrupts the nutrition supply of tumor cells and suppresses DNA/RNA synthesis (4). Therefore, this enzyme leads to cell growth inhibition, apoptosis, and autophagy induction in the neoplastic cells (5). All these specifications present L-asparaginase as a more effective therapeutic option in ALL treatment and an attractive research subject in other

cancer studies (6).

L-asparaginase is one of the enzyme products with high production levels worldwide, and it supplies about one-third of the world's need for anti-leukemia/lymphoma agents (7). In addition, the global demand for asparaginase will reach about USD 420 million by 2025 (8). Several formulations of L-asparaginase for industrial and clinical use are available in the market, including those of bacterial and native origin, polyethylene glycol (PEG), and recombinant L-asparaginases from *Escherichia coli*, such as native L-asparaginases from *Erwinia* strains (9).

Bioinformatics research has opened new avenues for optimizing and improving drugs and facilities to enable research and development of pharmacological software without laboratory costs. Therefore, investigation of the purposefulness of pharmaceutical structures and scientific comparisons are carried out to achieve better structures. Asparaginase is available in therapeutic lines derived from *E. coli* and *Erwinia chrysanthemi* sources. Although they are successful in the treatments, they have disadvantages such as drug resistance. Therefore, it is necessary to study the new origins of the production of asparaginase (7-8).

This study aimed to find structural differences and similarities between L-asparaginase enzymes produced from bacteria grown in different microenvironments. *Kibdelosporangium* sp. MJ126-NF4, *Thermococcus kodakarensis* KOD1, and *Bacillus subtilis* BEST7613 were selected to study L-asparaginase in mesophile, thermophile, and halophile strains.

Methods

Sequence analysis

The nucleotide and amino acid primary sequences of L-asparaginase derived from *B. subtilis* BEST7613, *Kibdelosporangium* sp. MJ126-NF4, and *T. kodakarensis* KOD1 were obtained from GenBank and NCBI (protein) databases. The EMBOSS Water pairwise sequence alignments were performed using ClustalW 1.83. Then, MEGA 7 software was used for analysis and to draw a phylogenetic tree of asparaginase-producing strains.

Structural analysis

Prediction of the secondary and tertiary protein structures of different L-asparaginases was carried out using SWISS-MODEL software. The TM-align software was used to align structures and compare tertiary protein structures. In addition, RasMol software was used to view PDB files.

Functional analysis

Protein domains of L-asparaginase originating from the three mentioned bacteria were analyzed using PROSITE software. In addition, theoretical isoelectric point (pI), molecular weight, and amino acid composition were predicated using the protein pI calculator (http://isoelectric.ovh.org/).

Results

The three bacterial strains had few similarities in primary nucleotide and amino acid sequences. As displayed in Table 1, there were differences in nucleotide and amino acid sequence lengths of L-asparaginase between the three strains of bacteria. Furthermore, using the EMBOSS Water tool of the ClustalW software, we evaluated the similarity rate of the primary nucleotide and protein structures of L-asparaginase in bacterial strains, as shown in Table 2. It should be noted that similarities and gaps were the comparative criteria for this analysis. Our analyses revealed that similarity rates of L-asparaginase nucleotide sequences were 42.8% to 44.7% in the three studied strains. Interestingly, the similarity rate of L-asparaginase amino acid sequences between *Kibdelosporangium* and *T. kodakarensis* strains was 45.2%. In addition, we found 49.9% and 41.5% similarity rates in amino acid sequences of L-asparaginase between *B. subtilis* and *Kibdelosporangium* or *T. kodakarensis*, respectively.

Phylogenetic analysis based on L-asparaginase's amino acid and nucleotide sequences showed a non-identical common ancestor among *B. subtilis*, *Kibdelosporangium*,

Table 1. Comparison of molecular weight and PI of L-asparaginase enzymes

Bacteria	Nucleotide sequence	Amino acid sequence	Isoelectric point	Molecular weight
Kibdelosporangium	999 nt	332 aa	5.088	34.68
Thermococcus kodakarensis	987 nt	328 aa	5.498	35.68
Bacillus subtilis	1128 nt	375 aa	5.95	40.1

The *P*-values for molecular weight and pI were 0.8 and 0.9, respectively.

Table 2. The nucleotide and amino acid pairwise analysis for three L-asparaginase-producing strains

Double compare	Nucleotide similarity	Amino acid similarity
Kibdelosporangium and *Thermococcus kodakernesis*	Similarity: 44.7% Gaps: 41.7%	Similarity: 45.2% Gaps: 10.0%
Kibdelosporangium and *Bacillus subtilis*	Similarity: 42.8% Gaps: 41.1%	Similarity: 49.9% Gaps: 13.0%
Thermococcus kodakarensis and *Bacillus subtilis*	Similarity: 44.5% Gaps: 41.9%	Similarity: 41.5% Gaps: 19.9%

and *T. kodakarensis*. After drawing a phylogenic tree using MEGA 7 software in the phylogenetic analysis, we observed a different racial relationship depending on the type of amino acid and/or nucleotide sequences between the three strains (Figure 1). In a phylogenetic analysis based on L-asparaginase nucleotide sequences, *B. subtilis* and *Kibdelosporangium* showed higher degrees of racial relationship, which means that they have a common ancestor, while *T. kodakarensis* is in an older evolutionary category in comparison to *B. subtilis* and *Kibdelosporangium*. However, in the phylogenic tree drawn based on the amino acid sequences, *Kibdelosporangium* and *T. kodakarensis* represented a common ancestor, and *B. subtilis* was in an older evolutionary category in comparison to the others.

The number and sequence of helix- and strand-secondary structures of L-asparaginase were not similar in the three mentioned strains. Analysis of predicted secondary structures indicated that there were 15 α-helixes and 15 β-sheets in *Kibdelosporangium* and 12 α-helixes and 14 β-sheets in *T. kodakarensis*. Moreover, L-asparaginase produced from the halophilic strain, *B. subtilis*, had 16 α-helixes and 15 β-sheets. These findings suggest that the three studied strains differ in L-asparaginase protein secondary structures.

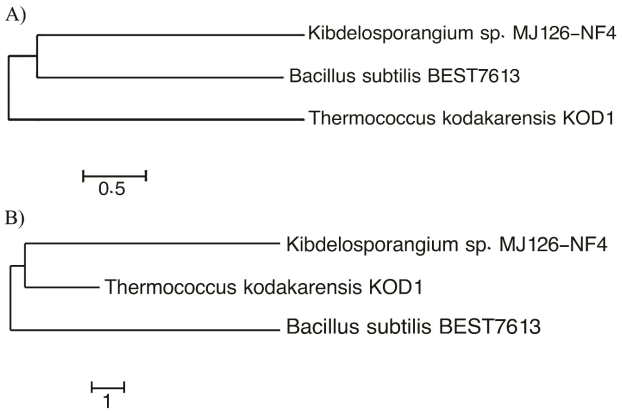

Figure 1. A phylogenic tree drawn using MEGA 7 software for three strains based on the nucleotide (A) and amino acid sequences (B)

Similarly, the tertiary structures of L-asparaginase were different in the mentioned strains. Given a similarity of less than 50% in nucleotide and/or amino acid sequences of L-asparaginase enzyme in the three studied strains, and regarding the differences in the evolution, construction, and/or composition of secondary structures, it is interesting to compare the tertiary structures of L-asparaginase in the three-studied strains. The analysis of the tertiary structures of L-asparaginase enzyme using SWISS-MODEL software showed that there are apparent differences in the tetrameric structures between the *B. subtilis*, *Kibdelosporangium*, and *T. kodakarensis* strains (Figure 2). Alignment analysis using TM-ALIGN indicated the differences among the tertiary structures of L-asparaginase enzymes in the three bacteria, as shown in Figure 3.

The amino acid frequencies of L-asparaginase originating from the mentioned strains differed. Using an isoelectric online tool, we analyzed the frequency of the amino acids in L-asparaginase in the three studied strains. Cysteine and selenocysteine, polar uncharged amino acids with sulfur and selenium, and tryptophan, a nonpolar amino acid, were rarely found in the structure of the three enzymes. However, the nonpolar alanine amino acid in *Kibdelosporangium* and *T. kodakarensis* and the nonpolar leucine amino acid were very frequent in *B. subtilis*. There were remarkable differences in the frequency of some amino acids, including alanine, proline, serine, asparagine, lysine, and arginine, in the protein structure of L-asparaginase in the three studied strains. Furthermore, tryptophan did not exist in *T. kodakarensis*. It would still be the reason for the differences in some physicochemical characteristics of L-asparaginase, such as half-life and protease sensitivity among the three bacteria.

There were no differences in the molecular weight and pI of L-asparaginase. Unexpectedly, no significant differences were found when comparing pI and molecular weight in the three studied protein structures using isoelectric tools.

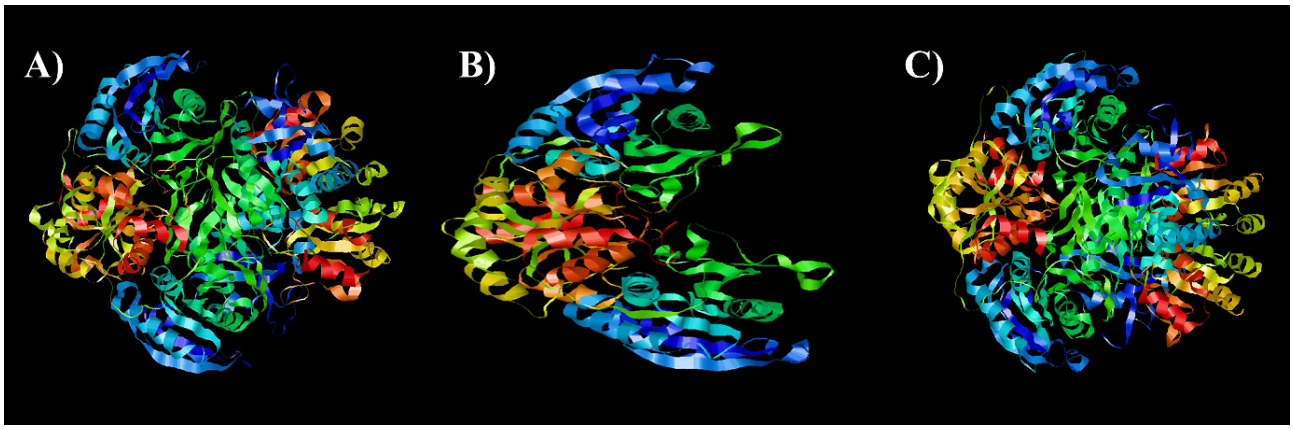

Figure 2. Analysis of L-asparaginase tertiary structures using SWISS-MODEL software. All images were prepared using RasMol software. The tertiary structures were aligned in the same direction from right to left. Structure similarities are evident in the form of an arc on the left side. A) *Kibdelosporangium*, B) *Thermococcus kodakarensis*, and C) *Bacillus subtilis*

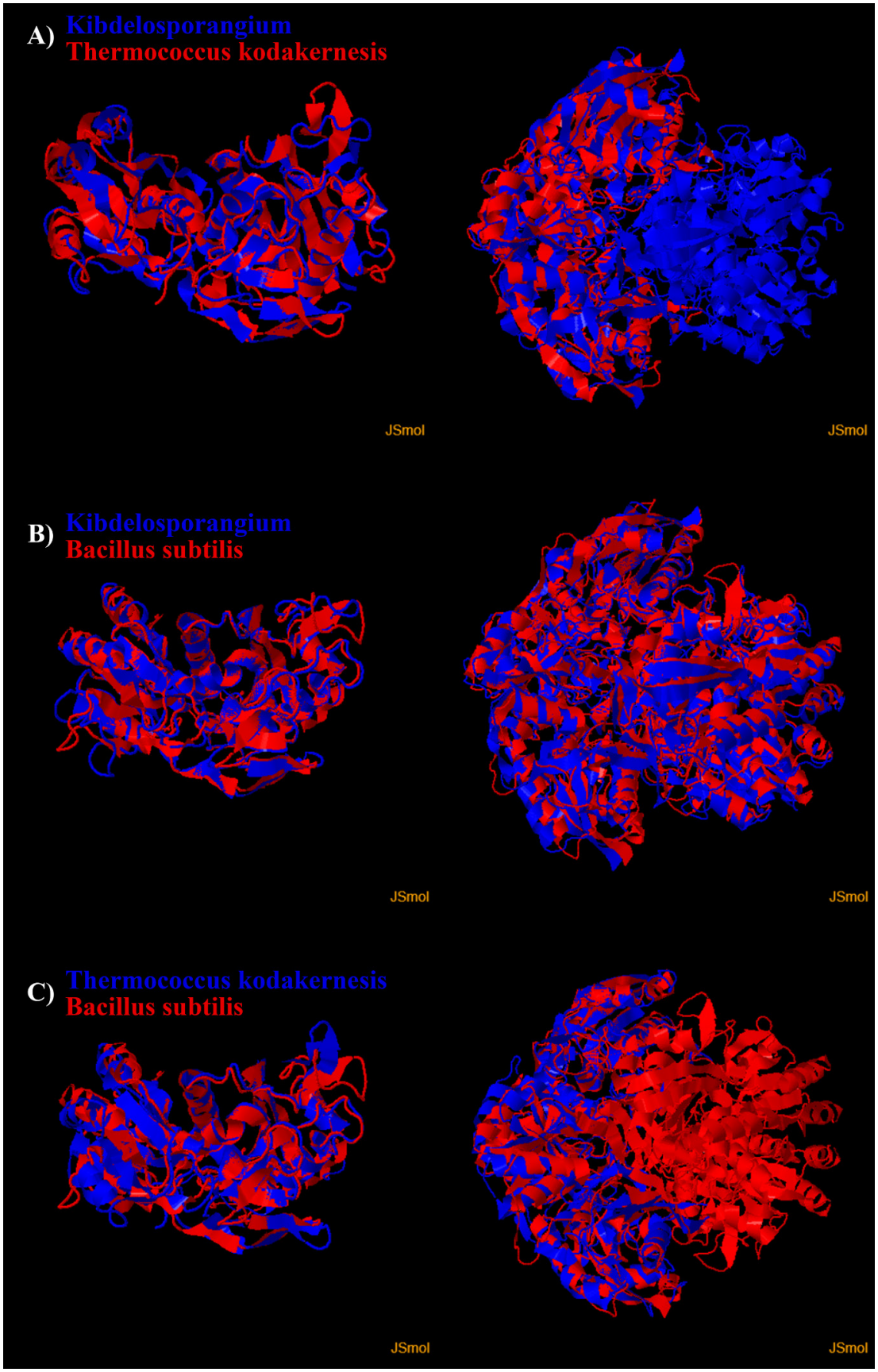

Figure 3. Comparison of L-asparaginase tertiary structures using the TM-ALIGN software in the three origins. Two different colors indicate the different tertiary structures of L-asparaginase in three investigated bacteria. The left images represent the overall alignment of two proteins, and the right images show the proteins with ligands and solvents. A) L-asparaginase tertiary structures in *Kibdelosporangium* (blue) and *Thermococcus kodakarensis* (red), B) L-asparaginase tertiary structures in *Kibdelosporangium* (blue) and *Bacillus subtilis* (red), C) L-asparaginase tertiary structures in *Thermococcus kodakarensis* (blue) and *Bacillus subtilis* (red)

As shown in Table 1, a bioinformatic comparison of these two parameters indicated that the molecular weight and pI of L-asparaginase enzymes extracted from *B. subtilis*, *Kibdelosporangium*, and *T. kodakarensis* strains were equal.

The asparaginase/glutaminase functional domain was common in the structure of L-asparaginase enzymes. The asparaginase/glutaminase domain was observed using PROSITE software in all three strains. However, the prokaryotic membrane lipoprotein lipid attachment site was only observed in *B. subtilis*, which may be due to specific conditions of the *B. subtilis* environment and a result of the evolutionary process in archaebacteria (Figure 4).

Discussion

The enzyme L-asparaginase (E.C.3.5.1.1) is used as an antitumor agent for the treatment of ALL (Figure 5). Various bacteria, including *E. coli*, *Proteus vulgaris*, *Erwinia carotovora*, *Pseudomonas fluorescens*, *Streptomyces karnatakensis*, *Saccharomyces cerevisiae*, *Serratia marcescens*, *Streptomyces venezuelae*, and some of fungi, including *Aspergillus*, *Penicillium*, and *Fusarium*, are used to extract the enzyme L-asparaginase (10). Among these, *E. coli* and *Erwinia carotovora* are the best sources for the extraction of this enzyme (11).

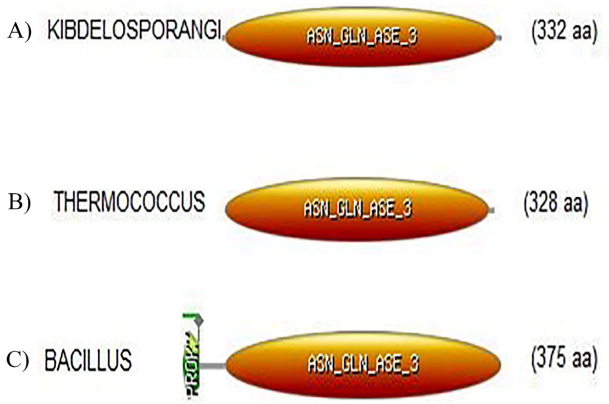

Figure 4. Analysis of L-asparaginase protein domains using the PROSITE database. The asparaginase/glutaminase domains were identical in all three strains, and the halophilic strain, *Bacillus subtilis*, has an additional prokaryotic domain

L-asparaginases showed disparate characteristics, because of the differences in growth medium, type of cell wall, and catalase activity of bacterial strains. Remarkable dissimilarity in the primary nucleotide and amino acid structures drew our attention to investigate other structures. Analysis of the secondary and tertiary structures, protein domains, and frequency of amino acids showed inconsistency in L-asparaginase structures between *B. subtilis*, *Kibdelosporangium*, and *T. kodakarensis*. Interestingly, all three strains had much similarity in functional parameters, including molecular weight, functional domain, and a pI of L-asparaginase. Hence, the similarity of these strains was probably due to protein structure and motifs. Conversely, these proteins were functionally similar, regardless of incompatibility in the primary nucleotide structure. Noticeable functional similarities in L-asparaginase isoelectric points, molecular weight, and functional domain at the macromolecular level, despite differences in the number and frequency of nucleotides and amino acids, confirm the codon usage theory and align with our understanding of the evolutionary process of proteins (12).

Nowadays, resistance to chemotherapy agents, such as L-asparaginase, is a major obstacle to successful cancer treatment (13). In addition, the researchers' purpose is to produce biotechnological drugs with a long half-life and protease-resistant sites. Due to the disparity in the frequency of amino acids in the structure of L-asparaginases extracted from the three bacteria, their physicochemical properties are very diverse (14). By recognizing the differences in the amino acid sequences of various L-asparaginases and target sites of proteases, it may be possible to design better anticancer drugs (15). Previous studies have shown that cysteine protease enzymes are involved in L-asparaginase resistance and non-responsiveness in children with ALL. The L-asparaginase extracted from *E. coli* and *Erwinia* strains is sensitive to cysteine protease enzymes, such as cathepsin B and asparaginyl endopeptidase (AEP), respectively

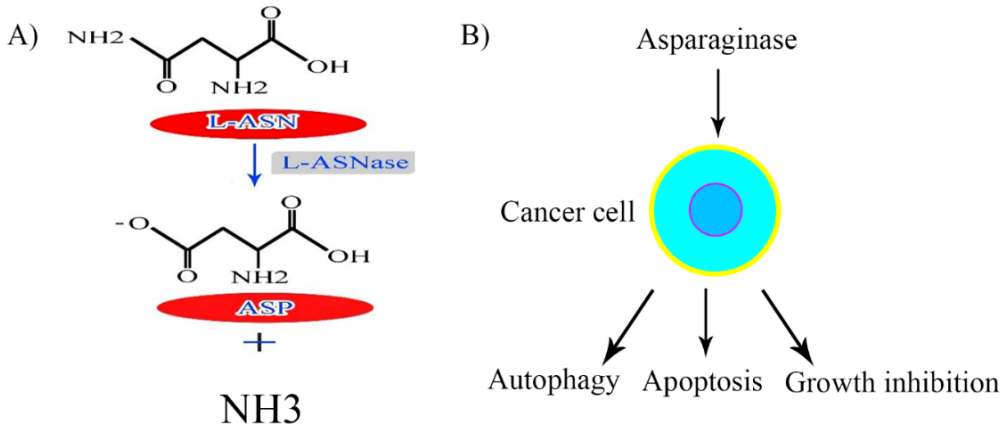

Figure 5. The scheme of enzymatic (A) and antitumor actions (B) of L-asparaginase

(16), (17). Simultaneously, the asparaginase we used in our study had no cysteine (in *Kibdelosporangium* and *T. kodakarensis*) or just one cysteine (in *B. subtilis*). In addition, as asparaginase has the highest stability at the pI of 6 in the blood (18), it seems that *B. subtilis*-derived asparaginase with a pI of 5.95 is more stable than *E. coli*-derived asparaginase with a pI of 5.72.

Furthermore, there is an inverse association between molecular weight and stability of proteins (19). Estimated molecular weights of L-asparaginases derived from *T. kodakarensis* and *Kibdelosporangium* are less than that of clinically used L-asparaginase, thus conferring them more stability in theory. Another factor is the number of alpha helices, which affects the protein stability (20). Due to the 13 alpha helices in its second structure, clinically used L-asparaginase would be less stable than the *Kibdelosporangium* and *B. subtilis*-derived types with 15 and 16 alpha helices, respectively. Therefore, it is suggested that the latter L-asparaginase enzymes would be suitable substitutes.

As asparaginase is present in many bacterial, fungal, and plant strains, any study requires the determination of study objectives. This study aimed to compare three different strains in terms of the habitat (halophile, mesophile, and thermophile). Therefore, this study is based on the structural dependence of asparaginase on its bacterial origin. Additionally, it aims to provide better alternatives for asparaginase producers, thereby promoting superior quality in both in vivo and in vitro settings.

Conclusion

In conclusion, bioinformatics data may help researchers select appropriate chemotherapy drugs with similar functions despite different structures. In addition, docking studies and analysis of enzymatic active sites can disclose diverse aspects of L-asparaginase protein structure in different strains and provide the best structure for designing the most effective medicine.

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Authors' Contribution

Conceptualization: Mohammad Mohammadzadeh-Vardin and Ali Mohammadi-Gollou.
Data curation: Ali Mohammadi-Gollou and Mohammad Mohammadzadeh-Vardin.
Formal analysis: Ali Mohammadi-Gollou.
Investigation: Ali Mohammadi-Gollou and Mohammad Mohammadzadeh-Vardin.
Methodology: Mohammad Mohammadzadeh-Vardin and Ali Mohammadi-Gollou.
Project administration: Mohammad Mohammadzadeh-Vardin.
Resources: Ali Mohammadi-Gollou.
Supervision: Mohammad Mohammadzadeh-Vardin.
Validation: Mohammad Mohammadzadeh-Vardin.
Visualization: Nowruz Najafzadeh, Farhad Jeddi, and Mohammad Mohammadzadeh-Vardin.
Writing–original draft: Ali Mohammadi-Gollou and Mohammad Mohammadzadeh-Vardin.
Writing–review & editing: Nowruz Najafzadeh, Farhad Jeddi, and Mohammad Mohammadzadeh-Vardin.

Competing Interests

The authors declared no conflicts of interest.

Ethical Approval

This study was approved by the Ethics Committee of Ardabil University of Medical Sciences with the ethical code IR.ARUMS.REC.1398.162.

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