Biochemical Characterization of Recombinant L-Asparaginase (AnsA) from *Rhizobium etli*, a Member of an Increasing Rhizobial-Type Family of L-Asparaginases

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We report the expression, purification, and characterization of l-asparaginase (AnsA) from *Rhizobium etli*. The enzyme was purified to homogeneity in a single-step procedure involving affinity chromatography, and the kinetic parameters Kₘ, Vₘₐₓ, and Kₘ/Vₘₐₓ for l-asparaginase were determined. The enzymatic activity in the presence of a number of substrates and metal ions was investigated. The molecular mass of the enzyme was 47 kDa by SDS-PAGE. The enzyme showed a maximal activity at 50°C, but the optimal temperature of activity was 37°C. It also showed maximal and optimal activities at pH 9.0. The values of Kₘ, Vₘₐₓ, kₐₚₖ, and kₐₚₖ/Kₘ were 8.9 ± 0.967 × 10⁻³ M, 128 ± 2.8 U/mg protein, 106 ± 2 s⁻¹, and 1.2 ± 0.105 × 10⁴ M⁻¹s⁻¹, respectively. The l-asparaginase activity was reduced in the presence of Mn²⁺, Ca²⁺, and Mg²⁺ metal ions for about 52% to 31%. In addition, we found that NH₄⁺, l-Asp, d-Asn, and β-aspartyl-hydroxamate in the reaction buffer reduced the activity of the enzyme, whereas l-Gln did not modify its enzymatic activity. This is the first report on the expression and characterization of the l-asparaginase (AnsA) from *R. etli*. Phylogenetic analysis of asparaginases reveals an increasing group of known sequences of the Rhizobial-type asparaginase II family.

Keywords: Type II asparaginase, *Rhizobium etli*, biochemical characterization

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1-L-Asparaginases (l-asparagine amidohydrolase, E.C. 3.5.1.1) are enzymes that catalyze the hydrolysis of l-asparagine to l-aspartate and ammonia. These enzymes are produced by a large number of microorganisms that include *Escherichia coli* [8, 9], *Erwinia cartovora* [41], *Enterobacter aerogenes* [46], *Corynebacterium glutamicum* [44], *Candida utilis* [33], *Vibrio succinogenes* ([17, 40]; formerly *Wolinella succinogenes*), *Thermus thermophilus* [48], and *Rhizobium etli* [47].

*R. etli*, the symbiotic host of leguminous plants, can use l-asparaginase as the sole carbon and nitrogen source through the action of two enzymes: l-asparaginase and l-aspartase [29]. In *R. etli*, two asparaginase activities were identified: a thermostable and constitutive asparaginase activity (named asparaginase I); and asparaginase II characterized by its thermostability, induced by asparagine, and repressed by the carbon source [30]. In the *R. etli* complete genome, two genes encoding l-asparaginase have been identified (Accession No. YP_468678 and AAF00929.1, respectively) [26, 47].

Based on amino acid sequences and biochemical properties, asparaginases can be divided into several families. The two larger and best characterized families include bacterial and plant asparaginases; the third family includes sequences with homology to *R. etli* asparaginase II [5]. The bacterial-type enzymes have been studied for over 30 years, mostly because they have shown beneficial pharmacological effect in the treatment of some types of lymphoblastic leukemias, among them the acute lymphoblastic leukemia (ALL) [6, 9, 25, 43, 50]. l-Asparaginases from *E. coli* and *E. chrysanthemi* are currently in clinical use in the treatment of ALL. Their therapeutic effect rarely occurs without some evidence of
toxicity, which in part is due to the glutaminase activity of these enzymes [2, 45]. In this sense, it is considered that L-asparaginases with high specificity for l-Asn and low-to negligible activity against l-glutamine are less troublesome during the course of anticancer therapy [16, 27, 37]. L-Asparaginase has been one of the most widely studied therapeutic enzymes [56]. Recently, several asparaginases have been thoroughly isolated and investigated in order to characterize enzymes with less toxic side effects [1, 21, 24, 35, 46]. Nowadays, the search for new asparaginase sources has been directed to the actinobacteria group of marine microorganisms [15, 51, 56].

Our interest in *R. etli* asparaginase II characterization arose from the fact that this enzyme is expressed when *R. etli* is grown in minimal media with asparagine as a sole carbon source, whereas when glutamine is used as a carbon source, asparaginase is not expressed [29]. In this sense, we hypothesized that AnsA might have a high specific activity for l-asparagine with negligible or absent glutaminase activity. In addition, its nucleotide and amino acid sequences reveal no relationship with asparaginases from *E. coli* and *E. chrysanthemi* that suggest different immunological specificity.

In this paper, we report the expression, purification, and biochemical characterization of recombinant L-asparaginase II (AnsA) of *R. etli*. Phylogenetic analysis among asparaginases is also discussed.

### Materials and Methods

#### Bacterial Strains, Plasmids, and Microbiological Procedures

The *Escherichia coli* strains used for subcloning were HB101 and XL1-Blue (Stratagene). *E. coli* strain BL21(DE3) (Novagen) was used for protein overexpression experiments. Growth and transformation of these strains were done by standard methods [52]. The plasmid used to overexpress the ansA gene was pTrcHis A (Invitrogen). pPIRL plasmid was used for supplying tRNA genes corresponding to the codons for arginine (AGG, AGA, and CGG), proline (CCC), and isoleucine (ATA) [4].

*E. coli* strains were grown in LB or 2XYT medium [52]. When needed, ampicillin was added at a final concentration of 100 µg/ml and chloramphenicol at 34 µg/ml.

#### Cloning of ansA and Construction of Plasmid pANSA

DNA manipulations were performed using standard techniques [52]. The ansA gene was amplified by PCR using plasmid pHZ11 as a template [45]. The 1.1 kb fragment of ansA was amplified using Platinum Taq DNA polymerase (Invitrogen) and oligonucleotides 5'-CCGGATCCATGGGAGAGAAATG-3' and 5'-CGGAATTCATCACGCGA-3'. These primers introduce restriction sites for BamHI and EcoRI, respectively (underlined). The PCR reaction was carried out with Platinum Taq DNA polymerase (Invitrogen). PCR conditions were as follows: initial denaturation for 5 min at 95°C followed by 35 cycles with denaturation at 98°C for 45 s, annealing for 30 s at 50°C, and elongation for 2 min at 72°C. A final step of 6 min at 72°C was done after the 35 cycles. The amplified fragment was cloned into the pUC18 plasmid (pUC-ANSA) for nucleotide sequence determination. The ansA gene was subcloned into the pTrcHisA expression vector (Invitrogen). The resulting recombinant plasmid pANSA was then used to transform the *E. coli* strain BL21(DE3) pPIRL.

#### Expression and Purification of AnsA

*E. coli* BL21(DE3)pPIRL cells harboring plasmid pANSA were grown in LB medium with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol, and protein expression was induced by 0.5 mM IPTG. Cells were lysed in a French press in buffer of 20 mM NaHPO₄ (pH 8.0), 0.4 M NaCl, and 1 mM phenylmethylsulfonyl fluoride. His-tagged AnsA was purified to homogeneity in a single-step procedure involving affinity chromatography using a FPLC Hi-Trap Chelating column (Amersham). The protein was eluted with an imidazole gradient (around 0.3 M). Fractions showing enzymatic activity were pooled and dialyzed against 30 mM KH₂PO₄ (pH 7.5) at 4°C, with five buffer changes at 2 h intervals each. Finally, the protein solution was dialyzed against the same buffer plus 50% glycerol, aliquoted, and stored at −70°C.

#### Assay of Asparaginase Activity

For the determination of asparaginase activity, the assay was performed under conditions previously established in the laboratory [18, 29]. Briefly, ammonium formation was performed in a 0.25 ml reaction mixture containing 30 mM KH₂PO₄ (pH 7.5), 10 mM L-asparagine, and 0.12 µg of the purified recombinant L-asparaginase, at 37°C for 2 min. Reaction was stopped by adding 50 µl of 5% trichloroacetic acid. The asparaginase activity was measured as the amount of ammonium produced, determined spectrophotometrically at 625 nm as described elsewhere [12]. Therefore, the coefficient of extinction (ε) from ammonia ion in a concentration range from 0 to 60 mM at 37°C, in 30 mM KH₂PO₄ (pH 9.0) was calculated by quadruplicate. The (ε) obtained was 21,874 M⁻¹ cm⁻¹.

One unit (U) of L-asparaginase activity was defined as the amount of enzyme that liberated 1 µmol ammonia min⁻¹. The specific activity was expressed as units of activity per milligram of protein. Protein concentration was determined at 25°C by the Bradford method [7] using bovine serum albumin (fraction V) as the standard. All experiments were conducted in triplicate.

#### Kinetic Parameters

The kinetic parameters of the AsnA were determined as above, using L-asparagine as substrate in a concentration range from 0 to 40 mM. The experimental data were adjusted to the Michaelis–Menten equation, thus obtaining the values of *Kₘ* and *Vₘₐₓ*. Data were analyzed using the software OriginLab version 8.5 (OriginLab Corporation, Northampton, MA, USA).

#### Effects of pH and Temperature

The effect of pH on L-asparaginase activity was determined at different values ranging from 6.0 to 11.0 at 37°C using buffers as described elsewhere [28]. The temperature of maximum activity was evaluated by incubating the enzyme for 2 min in a standard reaction mixture containing 30 mM KH₂PO₄ (pH 7.5) and 10 mM of L-asparagine at temperatures ranging from 25 to 60°C, with 5°C increments each. In addition, a second proof was carried out by incubating the enzyme at the temperatures of 27°C, 37°C, and 50°C in a reaction mixture as mentioned above, but at pH 9.0. The stability of
the enzyme was evaluated by incubating the purified enzyme at 50°C during different times ranging from 0 to 30 min with intervals of 5 min each. All experiments were carried out in triplicate.

**AnsA Substrate Specificity**

To determine the specificity of the enzyme by different substrates, the activity of AnsA for L-aspartate, L-asparagine, L-glutamine and L-glutamate was measured. The activity of AnsA was analyzed at 10 mM of each substrate and the reaction was followed for 10 min, performed in triplicate.

**Effects of Metal Ions and Asparagine Analogs**

The activity of the recombinant L-asparaginase was evaluated in the presence of various divalent cations (Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$), at concentration of 1 mM. All salts used were in the chloride form. The enzymatic activity was evaluated in reaction buffer containing 30 mM KH$_2$PO$_4$ (pH 9.0), 10 mM asparagine, and the corresponding metal ion, at 37°C, in triplicate. The effects of asparagine analogs and other substrates on the activity of the enzyme were also evaluated. L-Asp, NH$_4$Cl, L-Asn, L-Gln, and β-aspartyl hydroxamate at 5 mM were tested by inclusion of appropriate amounts in the assay mixture. All evaluations were carried out in the presence of L-asparaginase at 10 mM.

**Molecular Mass Determination**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 1.5 mm slab gel of 12% polyacrylamide (w/v). Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) was carried out to determine the molecular mass of the enzyme [38]. The protein bands were stained with Coomassie Brilliant Blue R-250. The standard proteins used for the molecular mass determination were the BenchMark Protein Ladder (Invitrogen) and High Molecular Weight Calibration Kit for Native Electrophoresis (Amersham).

**Sequence Searches and Alignments**

In order to retrieve members of the asparaginase II protein family, the asparaginase sequence of *Rhizobium etli* (NCBI access code: AAF00929) was used as query in a BLAST search [53] against the NR-database at the NCBI using an E-value ≤ 10$^{-6}$ as threshold. From this search, we identified a total of 372 protein sequences. Next, we excluded redundancy at 100% using the CD-suite program [39] and sequence fragments, defined as those sequences with coverage of ≤80% relative to the query sequence, leaving a total of 350 protein sequences. Alternatively, structural domains were identified using the PFAM database [23], in order to determine the domain architecture associated with these proteins. Subsequently, these sequences were aligned using ClustalW [54] utilizing the default parameters. In order to improve the alignment, the Profile option in the same program, considering the domain identified in PFAM (PF06089) as reference, was used. Finally, the alignment was manually inspected to exclude misalignments.

**Phylogenetic Analyses of Asparaginases**

Phylogenetic analyses based on protein sequences were carried out using the neighbor-joining (NJ) and maximum-likelihood method with the program of the PHYLIP software package [22]. One thousand replicates were performed in both analyses. For discussion, we present the NJ tree with bootstrap values ≥70%.

**RESULTS**

**Overexpression and Purification of L-Asparaginase**

Overexpression of recombinant His-tagged asparaginase into *E. coli* allows us to improve the enzyme yield and affinity purification process. The results of a typical purification run are summarized in Table 1; cell-free extracts of the *E. coli* transformants showed asparaginase activity of 1.109 U/mg protein. Once L- asparaginase was purified to homogeneity from cell-free extracts, the specific activity of the purified enzyme fraction increased to 20 U/mg protein. This means that the purification process recovered 97% of the enzyme from cell-free extracts (18-fold purification factor) in a single chromatographic step. Protein purity was evaluated by SDS-PAGE, which showed the presence of a single polypeptide chain of 47 kDa (Fig. 1A). The native molecular masses and oligomerization states of the purified L-asparaginase were established by Native-PAGE; a major band was found at 102 kDa when compared with molecular standard weight markers (Fig. 1B). The migrating position

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**Table 1. Purification progress of recombinant L-asparaginase from *Rhizobium etli***

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>100</td>
<td>110.9</td>
<td>1.109</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Hi-Trap-Ni</td>
<td>5.4</td>
<td>108</td>
<td>20</td>
<td>97</td>
<td>18</td>
</tr>
</tbody>
</table>

**Fig. 1. Purified recombinant L-asparaginase II from *Rhizobium etli***.  
(A) SDS–PAGE: M, molecular mass markers; lane 1, 5 µg of protein; lane 2, 10 µg of protein.  
(B) Native PAGE: M, native molecular mass markers; lane 1, 2 µg of protein; lane 2, 1 µg of protein. Protein bands were stained with Coomassie Brilliant Blue R-250.
as a single 102 kDa band in the gel strongly suggests the existence of homodimeric forms of the functional enzyme.

**Effects of pH and Temperature on Activity**

The pH optimum of AnsA was investigated under conditions previously standarized in the laboratory [18, 29]. The pH-dependent activity profile is given in Fig. 2, wherein it can be seen that the enzyme exhibited maximum activity at pH 9; the activity was retained at pH 9.5 up to 10.5 and after that the activity was reduced about 27% (pH 11.0). Additionally, it was observed that the specific activity of recombinant enzyme at alkaline pH (9.0 to 10.5) was at least 3-fold higher than at physiological pH (7.5).

As mentioned in Materials and Methods, the effect of temperature on enzyme activity was evaluated under three different conditions. The first assay was carried out at buffer conditions of pH 7.5; Fig. 3A shows the enzymatic activity at different temperatures ranging from 25°C to 60°C. The enzyme reached its maximum activity at 50°C, but at 60°C the enzyme began to lose its activity. Considering the above, we decided to evaluate the effect of temperature over the activity of the enzyme at pH 9.0, and found that the specific activity of the enzyme was lower at 27°C with respect to 37 and 50°C, with a briefly increment at 50°C (Fig. 3B). Considering the results of both assays, we suggest that pH rather than the temperature is the most important variable that modifies the enzymatic activity of AnsA. It is important to note that both plots show that the specific activity of AnsA at pH 9.0 was increased almost twice with respect to the maximum activity detected at pH 7.5. Finally, an experimental procedure was carried out to evaluate the thermal stability of the enzyme at the temperature of its maximum activity (50°C). In this case, the enzyme was preincubated at 50°C during different periods of time ranging from 0 to 30 min with 5 min intervals. After preincubation, the enzyme was place into the reaction buffer, and the formation of ammonium was registered.

**Fig. 2.** Effect of pH on the activity of purified *R. etli* L-asparaginase.

**Fig. 3.** Effect of temperature on the activity of purified *R. etli* L-asparaginase.

Preincubation of the enzyme for 10 min did not result in reduced activity; however, after 20 min, the activity was reduced more than 60% and its activity was lost after 30 min (Fig. 4).

**Fig. 4.** Heat inactivation of *R. etli* L-asparaginase.

The enzyme was preincubated at 50°C during different periods of time ranging from 0 to 30 min with 5 min intervals. Determination of enzymatic activity was carried out at pH 9.0.
The experimental data were fitted to the Michaelis–Menten substrate dependence. The turnover number $k_{\text{cat}}$ of enzyme. In this sense, the kinetic parameters for AnsA were investigated at various concentrations of the substrate, and $K_m, V_{\text{max}}$, and $k_{\text{cat}}$ for $\alpha$-asparaginase were determined. The enzyme showed typical Michaelis–Menten kinetics at lower substrate concentrations (Fig. 5) with the apparent $K_m$ and $V_{\text{max}}$ values for $\alpha$-asparaginase of $8.9 \pm 0.967 \times 10^{-3} \text{ M}$ and $128 \pm 2.8 \text{ U mg}^{-1} \text{ protein}^{-1}$, respectively. The turnover number $k_{\text{cat}}$ was $106 \pm 2 \text{ s}^{-1}$ and the apparent second-order rate constant ($k_{\text{cat}}/K_m$) was $1.2 \pm 0.105 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, which is related to the catalytic efficiency of the enzyme. In this sense, the kinetic parameters for AnsA of $R. etli$ were compared with those of other bacterial $\alpha$-asparaginases (Table 2). It is evident that $\alpha$-asparaginase from $R. etli$ hydrolyzes $\alpha$-asparagine at similar levels than $\alpha$-asparaginase from $E. coli$ and $E. carotovora$.

In regard to AnsA substrate specificity, enzymatic activity was not detected for $10 \text{ mM}$ $\alpha$-aspartate, $\delta$-asparagine, $\delta$-glutamine, and $\gamma$-glutamine as substrates. The activity of AnsA was specific for $\alpha$-asparagine. The above results confirm that AnsA does not show glutaminase activity (data not shown).

Effects of Metal Ions and Asparagine Analogs On $\alpha$-Asparaginase Activity
In order to evaluate the effects of some asparagine analogs and metal ions on the enzymatic activity, a series of experiments in the presence of $\alpha$-asparaginase in the reaction was carried out (Table 3). In relation to $\alpha$-Asn and $\beta$-aspartyl hydroxamate, it was observed at $5 \text{ mM}$ of concentration a reduction of enzymatic activity to approximately $61\%$ and $52\%$ with respect to full activation. In contrast, the presence of $\alpha$-Gln in the reaction maintained the enzymatic activity to levels similar to those of control reaction. Table 3 also shows the effects of various inorganic ions on activity; it was observed that $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, and $\text{Zn}^{2+}$ ions reduced the activity to about $62\%$ to $69\%$, whereas $\text{Mn}^{2+}$ affected the function by $50\%$.

In addition, AnsA was susceptible to inhibition by $\text{NH}_4^+$ and aspartic acid, the final products of enzymatic degradation of $\alpha$-asparagine. $\text{NH}_4^+$ showed a noncompetitive inhibitory effect, with a constant reduction of activity with respect to the control reaction with an apparent $K_i = 10 \text{ mM}$. Meanwhile, aspartic acid competitively reduced the enzymatic activity with an apparent $K_i = 33 \text{ mM}$ (data not shown).

Characteristics of Subfamily of Rhizobial-Type $\alpha$-Asparaginase II Enzymes
The amino acid sequence of AnsA (Accession No. AAF00929) from $R. etli$ was used for searching homologue proteins

<table>
<thead>
<tr>
<th>Organism</th>
<th>$K_m$ (M)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Rhizobium etli$</td>
<td>$8.9 \times 10^{-3}$</td>
<td>106</td>
<td>$1.2 \times 10^4$</td>
<td>Present work</td>
</tr>
<tr>
<td>$Escherichia coli$</td>
<td>$1.5 \times 10^{-5}$</td>
<td>24</td>
<td>$1.6 \times 10^6$</td>
<td>Derst et al. [14]</td>
</tr>
<tr>
<td>$Erwinia carotovora$</td>
<td>$8.5 \times 10^{-2}$</td>
<td>$31.4 \times 10^7$</td>
<td>$36.9 \times 10^7$</td>
<td>Kotzia and Labrou [34]</td>
</tr>
<tr>
<td>$Erwinia chrysanthemi$</td>
<td>$5.8 \times 10^{-5}$</td>
<td>$23.8 \times 10^7$</td>
<td>$41.2 \times 10^4$</td>
<td>Kotzia and Labrou [35]</td>
</tr>
</tbody>
</table>

Kinetische determinations for $10 \text{ mM}$ of $\alpha$-Asn as a substrate.

Table 2. Comparison of the kinetic parameters of $\alpha$-asparaginases of the organisms listed.

**Table 3. Effects of different metal ions and compounds on $\alpha$-asparaginase activity.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control$^*$</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>$\text{Ca}^{2+}$</td>
<td>1</td>
<td>69</td>
</tr>
<tr>
<td>$\text{Mg}^{2+}$</td>
<td>1</td>
<td>69</td>
</tr>
<tr>
<td>$\text{Mn}^{2+}$</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>$\text{Zn}^{2+}$</td>
<td>1</td>
<td>62</td>
</tr>
<tr>
<td>$\alpha$-Gln</td>
<td>5</td>
<td>96</td>
</tr>
<tr>
<td>$\delta$-Asn</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>$\beta$-Aspartyl hydroxamate</td>
<td>5</td>
<td>52</td>
</tr>
</tbody>
</table>

$^*$The specific activity of the control was $76.39 \text{ U mg}^{-1}$ protein. $\alpha$-Asparaginase activities were carried out by triplicate.
in the non-redundant database at the National Center of Biotechnology Information (NCBI). In previous analysis, the amino acid sequence of *R. etli* asparaginase II was grouped with seven other bacterial sequences, named the *Rhizobium etli* asparaginase family [5]. Therefore, the BLASTP search revealed 372 protein sequences with a significant score, but only 350 complete sequences were considered in this study (as described in Material and Methods). All proteins had a relative similar size (around 231 to 324 amino acids) and showed high significant similarity to AnsA (39% of average identity). The sequences obtained were aligned by the ClustalW program; the multiple sequence alignment (MSA) reveals high conservation between homolog proteins. From this alignment, it was possible to identify four conserved motifs along the sequences: at the N-terminal, the motifs RSx(2)KPxQA and ALxCASH; in the central position, NCSDKxHxGxL; and at the C-terminal position, the conserved motif AMx(3)Px(2)VAGxGRx(2)TxLM, which showed notable differences with the conserved regions of amino acid sequences of all known types I and II asparaginases [5]. Based on the MSA, a phylogenetic analysis was conducted to elucidate asparaginase relationships, by the neighbor-joining (NJ) method. The tree of these sequences showed four major branches that correlated with the bacterial taxonomical classification (Fig. 6 and Fig. S1). It is important to consider that the majority of members of this subfamily include microorganism that inhabit soil and marine ambient. One of the branches comprised actinobacterial enzymes. The second branch was composed of asparaginases

![Phylogenetic relationships among type II L-asparaginases.](image)

Fig. 6. Phylogenetic relationships among type II L-asparaginases.
An unrooted phylogenetic tree was calculated based on an MSA (described in Material and Methods). Type II asparaginases were grouped in four major branches that correlated with the bacterial taxonomical classification (see text for details).
from diverse bacterial classes, which included members of the Cyanobacteria, Actinobacteria, and Bacilli. In a third cluster, Alpha- and beta-proteobacteria (Rhizobiales and Rhodobacterales) enzymes were included. In the fourth cluster, Alpha- and beta-proteobacteria (Burkholderiales), Eukarya (Ascomycetes), Bacilli, and Deinococci were included. In this cluster, the R. etli asparaginase II was also included. It is important to note that this branch was closer to diverse eukarya, which suggests a horizontal gene transfer (HGT) event. These eukarya are mainly fungi and mosses. HGT events can also be suggested for diverse bacterial divisions, such as green nonsulfur and chlamydiae, among others; however, further analyses are necessary to corroborate these results.

**DISCUSSION**

Even though a significant number of bacterial asparaginase genes have been cloned, few studies had addressed the biochemical regulation of Rhizobial-type asparaginases. The present work was undertaken in order to characterize biochemically the recombinant L-asparaginase from R. etli. The described procedure for successful expression and purification yielded a very pure His-tagged protein in a simple chromatographic process. The high recovery of the protein by a single step may be attributed to the French press process for cell-free extract preparation instead of sonication. This procedure increased the concentration of His-tagged protein to be loaded to the chromatographic column and eliminated the bulk of contaminating proteins [36]. The His-tag was not removed from the purified enzyme, as it did not appear to affect the enzyme activity.

Whereas a tetrameric structure of the bacterial enzymes has been established for a number of type II L-asparaginases [11, 13, 17, 32, 55], a homodimeric form only has been reported for type 1 L-asparaginase from the hyperthermophilic archaeon *Pyrococcus horikoshii* [57]. L-Asparaginase II from *R. etli* showed an electrophoretic mobility in Native-PAGE of 102 kDa, which strongly suggests that the active form of the enzyme has a homodimeric organization; SDS-PAGE of the purified enzyme showed a pure homogenous band of 47 kDa.

AnsA from *R. etli* showed its maximum activity at pH 9.0 in 50 mM phosphate buffer, which is a value close to the range of better activity of other asparaginases purified from different microorganisms such as *E. coli* [10], *E. aroraeae* [55], *P. stutzeri* MB-405 [42], and other bacteria, which showed activity in pH’s ranging from 8.6 to 9.0 [3, 31]. The recombinant L-asparaginase showed that optimum activity was at 37°C, since the enzyme maintains its activity and stability as with other L-asparaginases. The temperature of maximum activity for the purified enzyme was 50°C; however, the enzyme lost its activity by more than 50% after 10 min of incubation, as was reported by *E. coli* L-asparaginase [49]. Therefore, the profile of activity at different temperatures and stability showed that the enzyme behaves like a typical mesophilic protein, since these parameters indicate a rapid and irreversible inactivation of AnsA.

The purified recombinant L-asparaginase showed a typical Michaelis–Menten profile in proximity to previously reported values for other asparaginases; it showed a value of *Km* lower than *E. carotovora* L-asparaginase [34], but two times higher than that previously determined by *E. chrysanthemi* and *E. coli* enzymes [14, 35]. In addition, the catalytic constant *kcat* of AnsA was higher than that of *E. coli* [19], but was at least 200-fold lower than that of *E. carotovora* and *E. chrysanthemi*, as shown in Table 2.

The substrate specificity of AnsA enzyme assays showed that only L-asparaginase and no glutaminase activity were observed, in contrast to the recombinant L-asparaginase of *E. carotovora* [36]. Based on the kinetic parameters similar to other bacterial L-asparaginases, but exhibiting a functional homodimeric catalytic form, it would be important to follow the characterization of the recombinant L-asparaginase from *R. etli*. Taken together, the AnsA biochemical properties are closed related with those reported for L-asparaginases used as therapeutic agents in the treatment of acute lymphoblastic leukemia (ALL). In regard to competition experiments, AnsA exhibited a traditional control of activity; it was negatively regulated by NH₄⁺ and aspartate, with inhibition constant (*Ki*) of 10 and 33 mM, respectively. Moreover, 5 mM D-asparagine showed inhibition by 40% with respect to full activity, suggesting possible binding of both substrates to the same catalytic site. In the presence of L-glutamine in the assay, L-asparaginase activity was maintained to levels similar to control reaction, which confirmed non additive activity and an absence of glutaminase activity, because L-Gln does not compete with L-Asn for the active site of L-asparaginase from *R. etli*. In contrast, the *Aspergillus niger* glutaminase–asparaginase does not show an additive activity on mixed substrate (L-Gln + L-Asn); however, considering the glutaminase activity of the enzyme, it suggests the competition of both substrates for the same catalytic site [21]. It is noteworthy that the purified enzyme AnsA does not present detectable glutaminase activity, which is very important, since glutaminase activity in therapeutic preparations of L-asparaginases has been implicated in causing side effects.

In summary, the glutaminase-free asparaginase activity of AnsA, and its biochemical properties discussed above, let us to propose AnsA II from *R. etli* as a potential enzyme for ALL treatment. Further studies are necessary to confirm its use as an anticancer therapeutic enzyme.

Finally, based on sequence comparisons, we found that type II asparaginases (where the Rhizobial-type family is included) is overrepresented in bacteria, with very few
homologs in eukarya. In this regard, it is possible that the homologs identified in eukarya are a consequence of horizontal gene transfer, based on the phylogenetic distribution of these enzymes and their abundance, less than 5% of the total asparaginases identified. From this, actinobacteria asparaginases represent 34.4% of the total proteins identified, whereas alpha-proteobacteria represent 26% of the total collection (see Table S1). Finally, the conserved motifs identified here are fully different to the previous consensus proposed, suggesting that this family has suffered a large number of changes as a consequence of their divergence evolution.

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