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Biochemical characterization and immobilization of Erwinia carotovora L-asparaginase in a microplate for high-throughput biosensing of L-asparagine

RUNNING TITLE:
Development an L-asparaginase biosensor

By

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Highlights

- L-ASNase from *E. carotovora* was cloned, expressed and characterized.
- The enzyme was used to assemble a microplate-based biosensor
- The biosensor was explored for the determination of L-Asn in biological samples
- The biosensor displayed high specificity and good stability

Abstract

L-asparaginases (L-ASNase, E.C. 3.5.1.1) catalyze the conversion of L-asparagine to L-aspartic acid and ammonia. In the present work, a new form of L-ASNase from a strain of Erwinia carotovora (EcaL-ASNase) was cloned, expressed in Escherichia coli as a soluble protein and characterized. The enzyme was purified to homogeneity by a single-step procedure comprising ion-exchange chromatography. The properties of the recombinant enzyme were investigated employing kinetic analysis and molecular modelling and the kinetic parameters (K_m, k_{cat}) for a number of substrates were determined. The enzyme was used to assemble a microplate-based biosensor that was used for the development of a simple assay for the determination of L-asparagine in biological samples. In this sensor, the enzyme was immobilized by crosslinking with glutaraldehyde and deposited into the well of a microplate in 96-well format. The sensing scheme was based on the colorimetric measurement of ammonia formation using the Nessler's reagent. This format is ideal for micro-volume applications and allows the use of the proposed biosensor in high-throughput applications for monitoring L-asparagine levels in serum and foods samples. Calibration curve was obtained for L-asparagine, with useful concentration range 10–200 μM. The biosensor had a detection limit of 10 μM for L-asparagine. The method's reproducibility was in the order of ±3–6% and L-asparagine mean recoveries were 101.5%.

ABBREVIATIONS USED:

BSA, bovine serum albumin; EcaL-ASNase, L-asparaginase from Erwinia carotovora; EcA, L-asparaginase from Escherichia coli; ErA, L-asparaginase from Erwinia chrysantemi, IPTG, isopropyl β-D-1-thiogalactopyranoside; L-Asn, L-asparagine; L-Asp, aspartic acid; L-Gln, L-glutamine; PAGE, polyacryamide gel electrophoresis; SDS, sodium dodecyl sulfate; Suc, succinic acid.

KEYWORDS: L-asparaginase, hydrolase, kinetic analysis, enzyme immobilization.

Introduction

L-ASNases are enzymes that primarily catalyze the conversion of L-asparagine (L-Asn) to aspartic acid (L-Asp) and ammonia [1]. They are also able to hydrolyze L-glutamine (L-Gln) but at a lower rate [2-9]. L-ASNases exist in many bacterial organisms, but only the enzymes from Escherichia coli (EcA) and Erwinia chrysantemi (ErA) have been used as chemotherapeutics in Acute Lymphoblastic Lymphoma (ALL) over the last three decades [2-9]. The L-glutaminase activity of EcA and ErA amounts to approximately 2 and 10%, respectively, of their L-asparaginase activity. Recent reports have shown that Erwinia carotovora L-ASNase, and two Helicobacter pylori L-ASNases have low glutaminase activity [10-14]. More recently, a robust L-ASNase having low-glutaminase activity from Bacillus licheniformis was characterized [15].

Bacterial L-ASNase are homotetramer enzymes. Each monomer is composed of ~330 amino acid residues, arranged in two domains: a large N-terminal domain and a smaller C-terminal domain. The two domains are linked by an approximately 20 residue flexible loop [5,16,17,18]. The active site of L-ASNase is located between the N-terminal and C-terminal domains of two adjacent monomers. Residues responsible for ligand binding form the rigid part of the active site [19-22]. The flexible part of the active site (residues 12-27) controls the entrance of the binding pocket [16,17,19-21].

The development of L-Asn biosensor attracts attention due to its potential use as a diagnostic tool for monitoring L-Asn levels in leukaemia samples and in foods [22-25]. L-Asn is the main amino acid that forms acrylamide (highly carcinogenic) in baked or fried food by reacting with reducing sugars at high temperature [1]. Therefore, the development of an L-Asn biosensor is both of academic and practical interest. So far, only a few works have been reported on the development of L-ASNase-based biosensors for the determination of L-Asn [22-25]. In the first example, the Archaeoglobus fulgidus L-ASNase was immobilized in front of an ammonium-selective electrode and used to develop a biosensor for L-Asn [22]. The biosensor had a detection limit of 6×10^{-5} M for L-Asn. In another example, the E. coli K-12 L-ASNase with phenol red indicator were immobilization on nitrocellulose membrane, silicon gel and calcium alginate beads. This biosensor was applied for the detection of L-Asn in normal and leukaemia serum samples [23]. In another example, a plant asparaginase-based asparagine biosensor for leukemia was developed [24]. The main drawback of the reported methods was the use of enzymes with both L-asparaginase and L-glutaminase activity. More recently the Leu90Ile mutant enzyme L-ASNase from E. carotovora (EcaL-ASNase) was used to assemble a cuvette-based biosensor L-Asn monitoring [25]. The main drawbacks of this previous method were the low L-asparaginase activity of the mutant enzyme (3.3 %) and its low stability.

In the present study, a new form of L-ASNase from Erwinia carotovora was cloned and expressed in E. coli as a soluble protein and the recombinant enzyme was characterized. The enzyme displays high L-asparaginase activity and low L-glutaminase activity, less than ~ 0.8 % of that of its L-asparaginase activity. These desired properties prompt us the application of this enzyme for the development of a biosensor specific for L-Asn. The enzyme was used to assemble a microplate-based biosensor suitable for high-throughput screening of biological samples (potato and human serum). The results of the present study provide new information into L-ASNases biochemistry and give further insights towards the development of a new application with potential use in food and clinical chemistry.

Materials

L-Asn, L-Asp and L-Gln were obtained from Serva (Heidelberg, Germany). Genomic DNA isolation kit, Nessler's reagent, N^{α}-Acetyl-L-Asn, succinamic acid and N^{β}-L-aspartyl-phenylalanine methyl esters were obtained from Sigma-Aldrich (USA). Crystalline bovine serum albumin (fraction V), were purchased from Boehringer Mannheim, (Germany). Nessler's reagent, D-Asn, β-alanine amide and GDH were obtained from Fluka (Germany). All other molecular biology reagents and kits were from Invitrogen (USA).

Methods

Cloning, expression, and purification of EcaL-ASNase

In an effort of finding alternative sources of L-ASNase we screened different strains of E. carotorova. The enzyme from E. carotorova 978 exhibited high L-asparaginase activity and low L-glutaminase activity and was selected for further study. The Erwinia carotovora strain 978 was grown at 30 °C in a medium containing 1% (w/v) peptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. After 24h, cells were pelleted by centrifugation and genomic DNA was isolated using a commercial kit (Sigma-Aldrich). PCR was used to amplify the full-length ORF from genomic DNA using the oligo primers synthesized to the 5' region of the Erwinia carotovora L-ASNase gene [10] (GenBank accession number AAS67027.1) from the ATG start codon (5'-ATGTTTAACGCATTATTCGTTGTGTTTTTG-3') and to the 3' end of the gene finishing at the TAA stop codon (5'-TTAAGCTTTTAATAAGCGTGGAAGTAATCC-3'). The PCR reaction was carried out in a total volume of 50 µl contained 8 pmol of each primer, 30 ng template genomic DNA, 0.2 mM of each dNTP, 5 µL 10 x Pfu buffer and 2 units of Pfu DNA polymerase. The PCR procedure comprised 30 cycles of 2.5 min at 96 ºC, 2 min at 46 ºC and 3 min at 72 ºC. A final extension time at 72 ºC for 10 min was performed after the 30 cycles. The PCR product was gel-purified and TOPO ligated into the expression vector pCR®T7/CT-TOPO®. The resulting expression construct pEcaL-ASNase was sequenced along both strands and was used to transform competent

E. coli Rosetta (DE3) cells in the presence of 100 μg/mL ampicillin. Transformed E. coli cells were grown at 37 ºC in 1 L LB medium containing 100 μg/mL ampicillin. The synthesis of EcaASNase was induced by the addition of 1 mM IPTG when the absorbance at 600 nm was 0.6-0.8. Six hours after induction, cells were harvested by centrifugation at 10,000 r.p.m. and 4 ºC for 20 min, resuspended in sodium phosphate buffer (50 mM, pH 7), sonicated, and centrifuged at 10000g for 20 min. The supernatant was collected and dialysed against 20 mM potassium phosphate buffer, pH 5.5. The dialysate was applied to a column of CM-Sepharose CL6B (2 mL, 1.5 x 1.5 cm I.D.) previously equilibrated with 20 mM potassium phosphate buffer, pH 5.5. Non-adsorbed protein was washed off with 50 mL equilibration buffer. Bound EcaL-ASNase was eluted with 20 mM potassium phosphate buffer pH 8.5. Collected fractions (1 mL) were assayed for L-asparaginase activity. The solution with purified enzyme was dialysed against 0.1 M potassium phosphate buffer and stored at -20°C in glycerol/0.1 M Tris/HCl buffer pH 7.5, 50/50 (v/v).

Assay of enzyme activity and assay for the determination of protein concentration

L-asparaginase activity was assayed by measuring the rate of ammonia formation using the Nessler's reagent [10]. L-glutaminase activity was measured by determining the rate of ammonia formation using the L-asparaginase/glutamate dehydrogenase coupled reaction assay [10]. One unit of L-ASNase activity was defined as the amount of enzyme that liberates 1 μmol of ammonia from L-Asn per min at 25°C. Protein concentration was determined at 25°C by the method of Bradford (1976) using bovine serum albumin (fraction V) as standard.

Kinetic analysis

Steady-state kinetic measurements were performed in 0.1 M Tris-HCl buffer, pH 8.0, by varying the concentration of the substrate (L-Asn, D-Asn, L-Gln, N^{α}-acetyl-L-Asn, succinamic acid, β-alanine amide). The kinetic parameters k_{cat} and K_m were calculated by non-linear regression analysis of experimental steady-state data. Kinetic data were analyzed using the computer program GraFit (Erithacus Software Ltd., [26]). .

Bioinformatics analysis and molecular modelling

The molecular model of EcaASNase was constructed using SWISS-MODEL [27]. The determined X-ray crystal structures of E. carotovora enzyme (PDB codes 2JK0.1 and 2HLN.1; 98.70% sequence identity) were used as templates. GMQE (Global Model Quality Estimation) overall scores were used to choose the final model. Analysis of packing, solvent exposure and stereochemical properties suggested the final enzyme model to be of very good overall quality. For inspection of models and crystal structures we used the program PyMOL [28]. The theoretical isoelectric point and molecular mass

of the enzyme were calculated using the ExPASy - ProtParam tool (http://web.expasy.org/protparam/) [29].

Thermal stability of the wild type and mutant enzymes

Thermal inactivation of the wild-type enzyme was monitored by activity measurements. Samples of the enzyme, in 10 mM KH_2PO_4 buffer pH 7, were incubated at different temperatures (20 ºC to 60 ºC) for 7.5 min. Subsequently the samples were assayed for residual activity. The half inactivation temperature (T_m) was determined from the plots of relative inactivation (%) versus temperature (°C). The half inactivation temperature (T_m) is the temperature at which 50% of the initial enzyme activity is lost after heat treatment. The stability test was repeated in three separate experiments.

Enzyme immobilization

The enzyme was immobilized by cross-linking with glutaraldehyde and bovine serum albumin (BSA) [25,30-34]. Aiming at the determination of the optimal immobilization conditions concerning the concentrations of bovine serum albumin (BSA), glutaraldehyde and enzyme, different concentrations were evaluated. All conditions were tested as following: the enzyme and the BSA were initially mixed, followed by the rapid addition of glutaraldehyde and additional mixing. Subsequently, the resultant mixture was and deposited into the well of a microplate in 96-well format where it was left to solidify for 45 min at 25 °C. When the mixture was solidified, it was rinsed twice with 100 mM Tris/HCl buffer, pH 8.0 to remove excess of reactive compounds. The activity of the immobilized enzyme was measured after the addition in each well 100 mM Tris/HCl buffer, pH 8.0 and substrate (L-Asn or L-Gln). The activity of the immobilized enzyme was measured after incubation for 30 min. Following incubation, the upper solution from the well (10-50 μL) was removed and transferred to a new microplate well, where Nessler's reagent (50 μL) and ddH_2O (to adjust the total volume to 200 μL) were added. The mixture was incubated for 5 min and the absorption was measured at 436 nm. The total assay time was 35 min. The condition which exhibited the highest immobilized enzyme activity was chosen for further studies. In parallel, free enzyme of the same concentration as that of the immobilized setup was equally treated (incubated in 100 mM Tris/HCl buffer, pH 8.0 and substrate) and served as control for direct comparison with the stability of the immobilized enzyme.

The immobilized enzyme system was used for the determination of L-Asn in potato samples as described previously [20]. Potato sample (20 g, obtained from the local market) were extracted (mortar and pestle) with water (40 mL) containing 2% (w/v) polyvinylpolypyrrolidone (PVPP) at 25°C. Perchloric acid (1 M, 8 mL) was added to the extract and incubated at room temperature for 30 min. The extract was neutralised (KOH, 1M) and centrifuged to separate insoluble material. An aliquot from the supernatant (100-

200 μL) was used to analyse L-Asn. Normal blood serum were used the determination of L-Asn without any pretreatment. Validation of the results for L-Asn determination was carried out using the procedure described in a commercial kit (www.megazyme.com).

Results & discussion

Characterization of recombinant EcaL-ASNase

PCR was used to amplify the full-length sequence of L-ASNase from E. carotovora 978 genomic DNA. The PCR product was cloned into the T7 expression plasmid pCR®T7/CT-TOPO to give the pEcaL-ASNase plasmid. Sequencing analysis reveals an ORF that encodes for a polypeptide of 346 amino acids. BLASTp search showed that this sequence shares 96-99% identity with other L-ASNases from E. carotovora species (Figure 1). The important amino acid residues (Ser60, Glu61, Asp94, Thr13, Ala118, numbering according to PDB file 2JK0) that form the active-site region and play important role in substrate binding and catalysis are all conserved in the EcaL-ASNase sequence. The flexible loop of the active site (residues 13-28) that controls access to the binding pocket and carries the catalytic nucleophile Thr13 is conserved. A putative signal peptide (residues 1–24) to direct enzyme's transport across the periplasmic membrane was found at the N-terminal end of the in EcaL-ASNase sequence. The predicted isoelectric point and molecular mass of the mature polypeptide (residues 25-346) were estimated 8.85 and 34,075.95 Da, respectively. The pEcaL-ASNase plasmid was used to transform the expression host E. coli Rosetta (DE3). E. coli transformants showed high L-asparaginase activity with a specific activity of 12.5 U/mg protein. EcaL-ASNase was purified in a single-step procedure using cation-exchange chromatography on CM-Sepharose ion-exchanger. The enzyme was absorbed at pH 5.5 and eluted at pH 8.5. The purity of the final EcaL-ASNase preparation was evaluated by SDS-PAGE, which showed the presence of a single polypeptide chain (Figure 2).

The recombinant EcaL-ASNase was subjected to kinetic analysis and k_{cat}, and K_m parameters for a number of substrates were determined by steady-state kinetic analysis (Table 1). The K_m values for L-Asn and L-Gln were measured as 0.07 mM and 5.2 mM, respectively. The enzyme exhibits low L-glutaminase activity, which is about 0.8% of that of its L-asparaginase activity. The L-glutaminase activity is lower than that exhibited by the E. coli and E. chrysanthemi enzymes (i.e. ~10%). Kinetic analysis using other similar amides showed that the enzyme does not hydrolyze β-alanine amide (descarboxyl asparagine), while succinamic acid (desamino-asparagine) is hydrolyzed although with significant lower efficiency, indicating that the enzyme displays an absolute requirement for a free α-carboxyl group but not for α-amino group in the substrate.

The molecular model of EcaL-ASNase was constructed, using homology modelling, to put the sequence and kinetic data in a structural context. The different amino acid found in the sequence of EcaL-ASNase compared to the crystallographic determined structure (Figure 1) are located in regions of no structural significance. Most of them are completely exposed to the solvent and do not participate in any significant molecular interactions. Analysis of the EcaL-ASNase model in complex with L-Asp and L-Glu suggested that the α-carboxyl group of the ligand interacts with the enzyme in a conserved manner (Figure 3). One of the oxygen atoms interacts with the amide group of Asp113 and with the γ-OH group of Ser79. The other oxygen atom forms a hydrogen bond with the amide group of Ser79 (Figure 3). The presence of an α-amino group in the substrate (Table 1) is also important but not essential for catalysis, as indicated by the ability of the enzyme to hydrolyze N^{α}-acetyl-L-Asn and succinamic acid at a lower rate, compared to that for L-Asn. Presumably, the presence of the α-amino group in the substrate contributes to its proper orientation in the active-site and therefore affects the catalytic efficiency of the enzyme. The α-NH_3^+ group of the ligand participate in two conserved hydrogen bonds formed with the carboxyl oxygen atoms of Asp113 and Glu80 (Figure 3).

The thermal stability of EcaL-ASNase was assessed by measuring their residual activity after heat treatment at various temperatures (20-60 ºC, Figure 4). The T_m was measured equal to 43.6±1.5 °C, suggesting that the enzyme displays moderate thermal stability. Similar published studies, carried out with other Erwinia-derived enzymes showed that the E. carotovora enzyme exhibits lower structural stability compared to the E. chrysanthemi enzyme. For example, Papageorgiou et al., (2008) [16] using denaturing experiments showed that E. carotovora L-ASNase has decreased thermodynamic stability as compared to the E. coli enzyme and is rapidly inactivated in the presence of urea.

The basis of a microplate-based biosensor for L-Asn monitoring

Desired properties for the use of L-ASNases for the development of biosensor include high L-asparaginase and low L-glutaminase activity. The high specificity towards L-Asn observed for EcaL-ASNase prompts us to investigate the development of a biosensor for the determination of L-Asn. In this sensor, the enzyme was immobilized by crosslinking with glutaraldehyde [25,30-34] and deposited into a well of a microplate in 96-well format. This format is ideal for micro-volume applications and allows the use of the proposed biosensor in high-throughput applications. Glutaraldehyde is a bifunctional reagent that binds proteins through their free amino groups, thus forming a three-dimensional network [30]. The reaction of amino group with glutaraldehyde forms the so-called Schiff base [25,30-34]. BSA was used in excess and reacting itself with glutaraldehyde played the role of the carrier within which the enzyme was immobilized. Table 2 shows the conditions which were experimentally screened for the determination

of the condition with the highest immobilization yield. Immobilization yield is defined as the ratio of the enzymatic units prior the immobilization to those measured after the immobilization. As shown in Figure 5, condition No 5 which contained 1% (v/v) glutaraldehyde, 20 mg BSA and 10 units of L-ASNase displayed the highest immobilization yield (78%). This condition was selected for further study. The selectivity of the biosensor was tested using L-glutamine and D-Asn. It is noteworthy that the immobilized enzyme did not show any activity using L-Gln or D-Asn as substrates.

The immobilized enzyme-catalyzed L-Asn hydrolysis results in concomitant release of NH_4^+ ions. The concentration of released ions reflects the progress of the L-Asn hydrolysis and can be measured using the Nessler's reagent. Linear calibration curve was measured within concentration range 10–200 μM and using the response obtained 30 min after addition of the substrates (Figure 5B). The observed linearity range is suitable for the determination of L-Asn in several biological samples such as cheese, juices, asparagus and human serum [35-38]. The reproducibility of sensor response to substrate, expressed by RSD, was in the order of ±3–6% (N=5). Recovery experiments using samples spiked with known amounts of L-Asn, showed that L-Asn recoveries ranged between 84.2 and 112%, with an average value of 101.5% (N=5) (Table 3).

Stability of the immobilized enzyme

The stability of the immobilization enzyme at 4 °C is shown in Figure 6. As can be seen from the figure, under the same storage temperatures, the activity of the immobilized enzyme decreased at a slower rate compared to that of the free enzyme. The immobilized enzyme retained about 50% of its initial activity after about 30 days, while the free enzyme totally lost its activity after eight days of incubation. Those data clearly suggest that, the immobilization benefited significantly the long-term stability of the enzyme, thereby making the use of glutaraldehyde-BSA immobilization chemistry suitable for the stabilization of L-ASNase.

An average of 20 cycles of analysis of samples containing L-Asn could be made until total deterioration of the sensor. The sensor was considered to be non-functional when its enzyme activity had fallen to about 60% of its original value about twenty days after enzyme immobilization.

Determination of L-Asn in potato and human serum samples

The EcaL-ASNase-based microplate biosensor was evaluated for the determination of L-Asn in potato and human serum samples. The mean L-Asn levels in different potato samples measured between 720±35μg/g to 1560±45μg/g fresh weight. These values are in agreement with the value for L-Asn in potato samples reported in the literature [35]. The biosensor was also used for the determination of L-Asn levels in normal blood serum

samples. The mean L-Asn levels in human serum samples was found 44.4±4.1 mM, which in agreement with the value for L-Asn in normal serum samples reported in the literature (32–56 mM), [38]. The results were further validated and showed very good reproducibility (error <5-10%) using the procedure described in a commercial kit (www.megazyme.com) for L-Asn determination in food samples. The kit is based on L-asparaginase/glutamate dehydrogenase coupled reactions.

Conclusions

In this study a new L-ASNase from E. carotovora has been cloned, expressed and characterized. The enzyme displays high specificity towards L-Asn and was used for the development of microplate-based biosensor for L-Asn determination. The proposed microplate biosensor offers a new method for the specific, convenient, cost effective and rapid measurement and analysis of L-Asn. The immobilized enzyme displayed good stability upon storage which significantly improves the practical viability of this analytical tool.

Conflict of interest

None

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Figure LEGENDS

Figure 1. Sequence alignments of EcaL-ASNase with type II L-asparaginases from E. carotovora species (NCBI accession numbers: KHN53959.1, WP_051906112.1, WP_043881724.1). The sequence of the crystallographic determined structure of L-ASNase from E. carotovora (PDB code 2JK0 [16]) was included in the alignements. The alignments were produced using Clustal Omega [39]. The secondary structure of L-ASNase from E. carotovora (PDB code 2JK0) is shown above the alignment. Alpha helices and beta strands are represented as helices and arrows, respectively, and beta turns are marked with TT. A column is framed if more than 70 % of its residues are similar according to physicochemical properties. The figure was created with ENDscript [40].

Figure 2. SDS-polyacrylamide gel electrophoresis of EcaL-ASNase purification on CM-Sepharose. Protein bands were stained with Coomassie blue G250. Lane 1, molecular weight markers; Lane 2: E. coli Rosetta (DE3) crude extract after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG); Lane 3: eluted EcaL-ASNase from CM-Sepharose.

Figure 3. Structural representations of the EcaL-ASNase model produced by homology modelling. (A): Ribbon representation of the modelled tetrameric structure of EcaL-ASNase in complex with L-Asp. L-Asp is shown in spacefill representation and colored according to atom type. **(B):** Diagram of the active site of the EcaL-ASNase model with L-Asp bound. L-Asp is shown in spacefill representation and colored according to atom type. Important amino acid residue that contributes to L-Asp binding (Thr13, Ser60, Glu61, Asp94, Ala118) are depicted in a stick representation, colored according to atom type and labelled. **(C):** Diagram of the active site of the EcaL-ASNase model with L-Glu bound. L-Glu is shown in spacefill representation and colored according to atom type. Important amino acid residue that contributes to L-Glu binding (Thr13, Ser60, Glu61, Asp94, Ala118) are depicted in a stick representation, colored according to atom type and labelled.

Figure 4. Thermal inactivation curve of EcaL-ASNase. The residual activities of the enzyme were measured after heat treatment for 7.5 min at various temperatures (20-60 °C). Each point represents the average of three determinations. The enzyme assay was carried out at 25 °C as described under "Methods". Activity of the unincubated sample was taken as 100%, and the percent of the remaining activity of the heated samples was calculated.

Figure 5. (A): The effect of glutaraldehyde (% v/v), bovine serum albumin (mg) and EcaL-ASNase (enzyme units) on immobilization yield (%). The conditions are listed in Table 2. **(B)**: Calibration curve obtained for L-Asn using immobilized EcaL-ASNase. Each point represents the average of three determinations.

Figure 6. The effect of immobilization on enzyme stability assessed by isothermal inactivation at 4°C. Stability of the free (●) and immobilized EcaL-ASNase (■). The enzyme assay was carried out at 25 °C as described under "Methods".

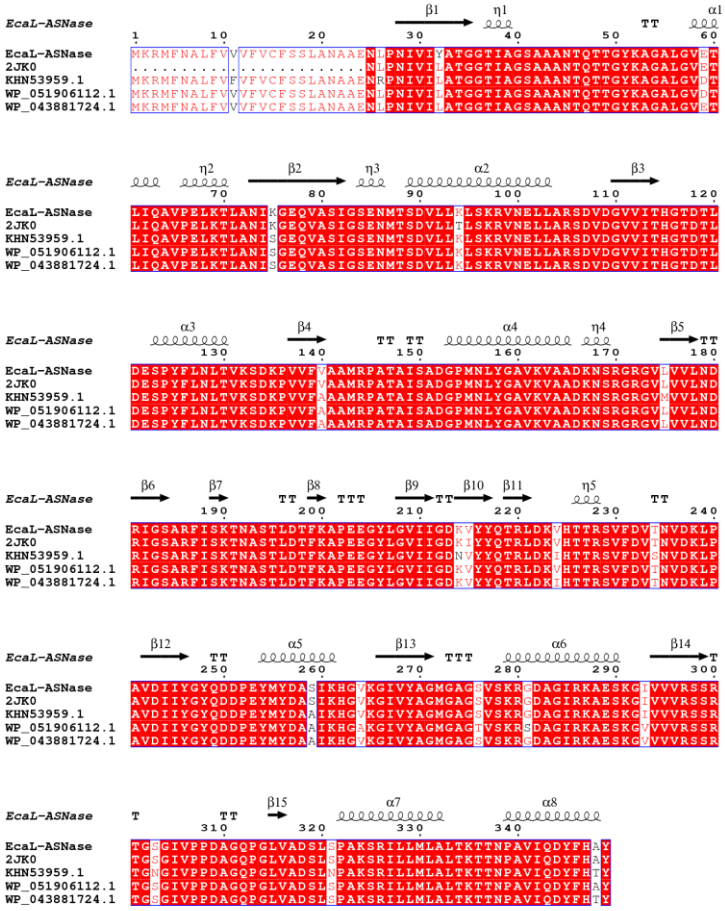


Figure 1

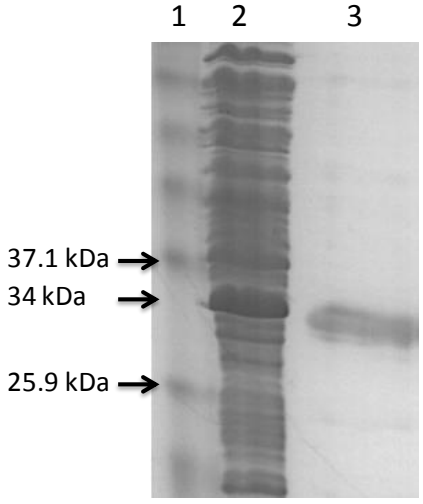
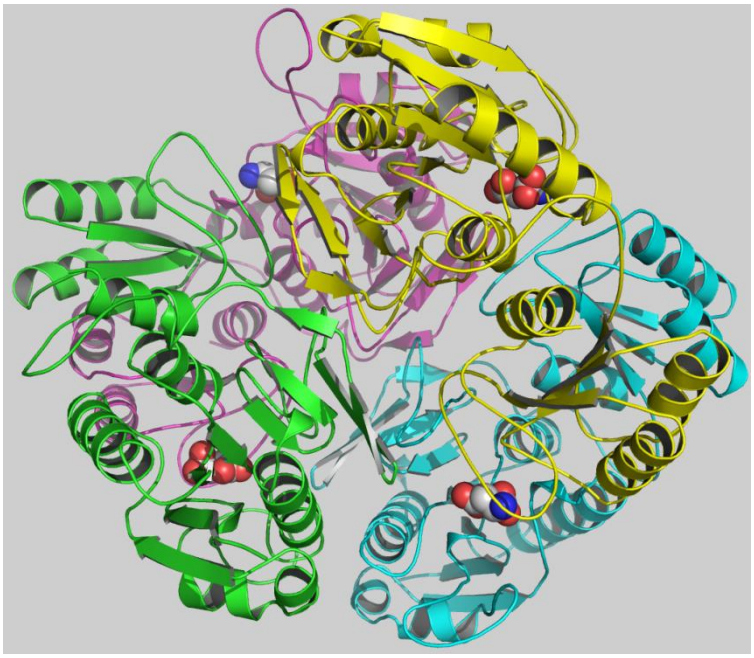


Figure 2

A



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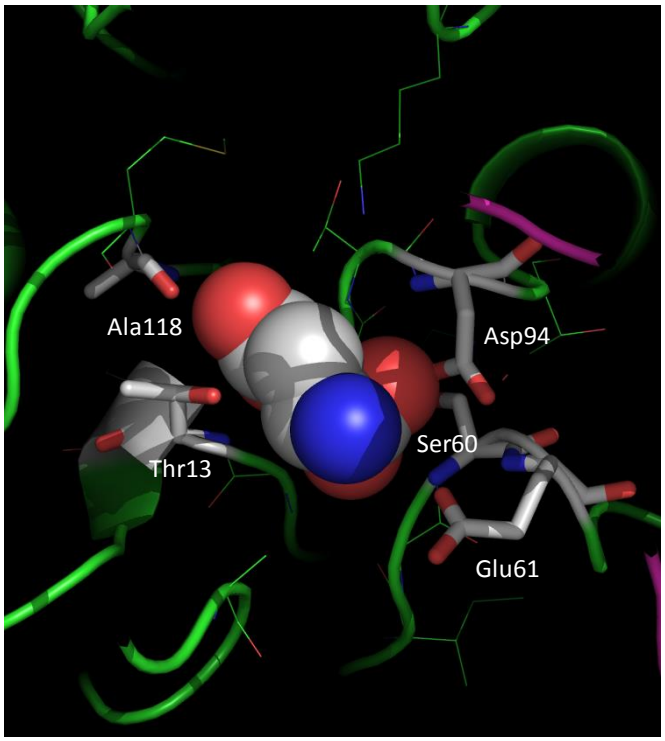

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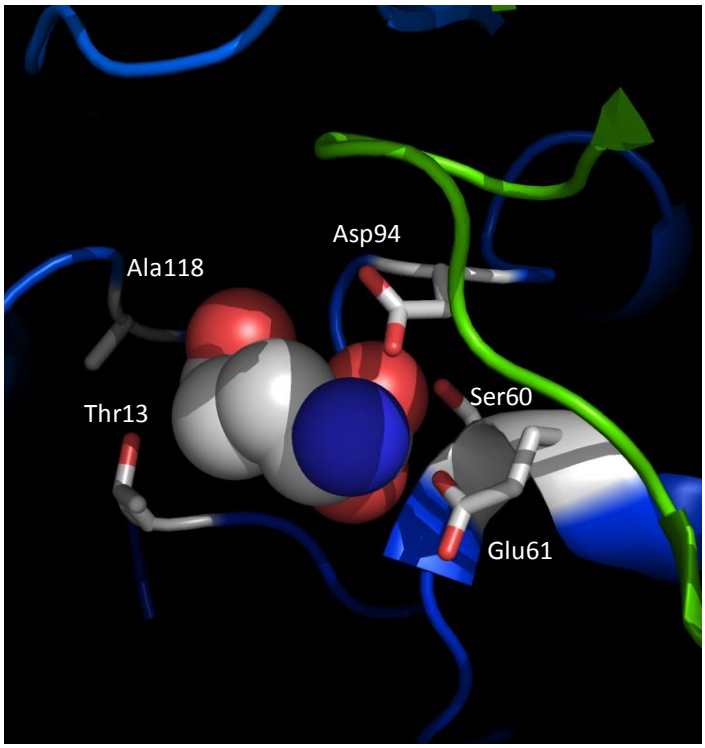


Figure 3

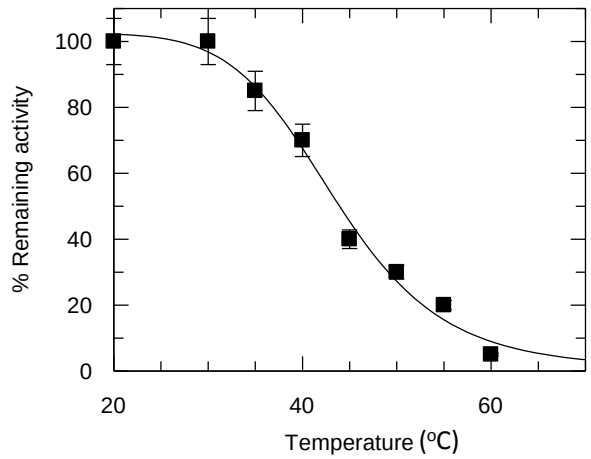
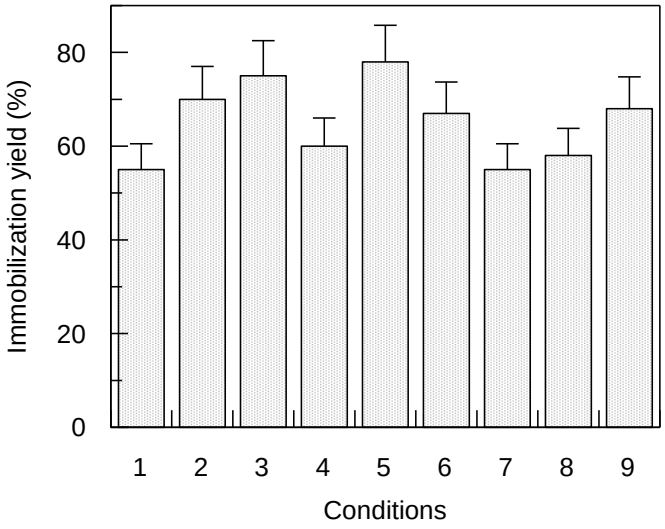


Figure 4

A



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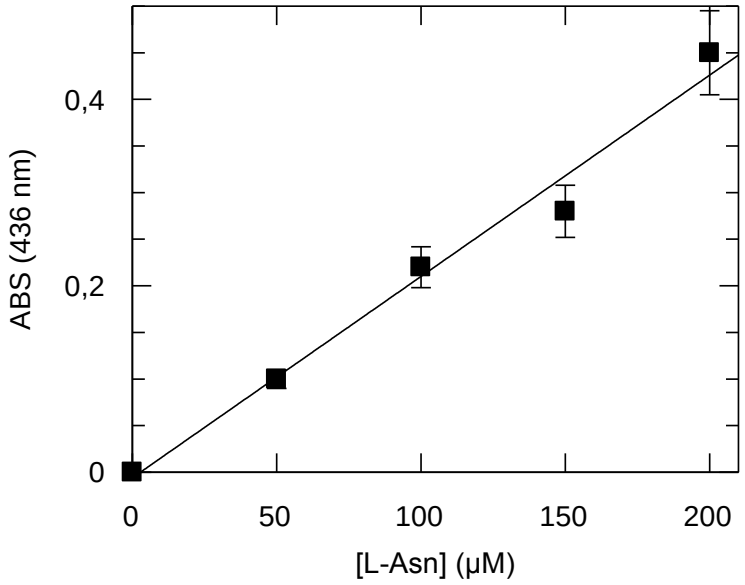

Figure 5

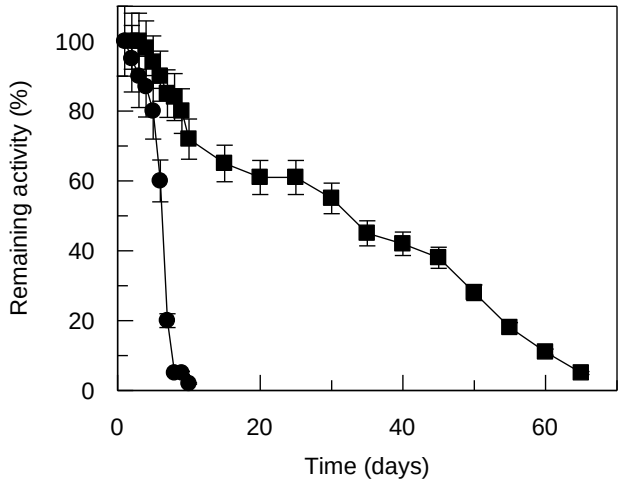

Figure 6.

Table 1. **Kinetic parameters of *Eca*L-ASNase.** All initial velocities were determined in triplicate. The kinetic parameters k_{cat} and K_m were calculated by non-linear regression analysis of experimental steady-state data using the GraFit (Erithacus Software Ltd.) program.

Substrate	**K_m (mM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m ($mM^{-1}s^{-1}$)**
L-Asn	0.07 ± 0.01	55.2 ± 2.2	788.6
L-Gln	5.2± 0.4	0.42 ± 0.1	0.42
D-Asn	45.2 ± 4.6	1.2 ± 0.2	0.03
Succinamic acid	0.3 ± 0.02	4.1 ± 0.2	13.7
N^{α}-Acetyl-L-Asn	0.5 ± 0.04	4.1 ± 0.2	8.2
β-Alanine amide	ND	ND	ND

Table 2. Tested concentrations of glutaraldehyde, BSA and enzyme for the determination of the optimal immobilization conditions.

Conditions (#)	**Glutaraldehyde (% v/v)**	**BSA (mg)**	**Enzyme (Units)**
1	0.5	10	5.0
2	0.5	20	10.0
3	0.5	30	20.0
4	1.0	10	5.0
5	1.0	20	10.0
6	1.0	30	20.0
7	1.5	10	5.0
8	1.5	20	10.0
9	1.5	30	20.0

Table 3. L-Asn and L-Gln recovery experiments in real water samples spiked with known amounts of each amino acid**.**

Sample ID	**L-Asn**			**L-Gln**		
	Added (µM)	**Found (µM)**	**Recovery (%)**	**Added (µM)**	**Found (µM)**	**Recovery (%)**
1	10	11.2	112	100	0	-
2	50	42.1	84.2	500	0	-
3	100	110.1	110.1	1000	0	-
4	150	142.2	94.8	1500	0	-
5	200	212.4	106.2	2000	0	-