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# Engineering substrate specificity of *E. carotovora* L-asparaginase for the development of biosensor

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ABSTRACT

L-Asparaginase (E.C.3.5.1.1, L-ASNase) is an enzyme extensively used as an anti-neoplastic agent in the chemotherapy of acute lymphoblastic leukemia (ALL). In the present study, we report the use of *in vitro* directed evolution for the creation of a new L-ASNase variant lacking glutaminase activity. A library of enzyme variants was constructed by staggered extension process (StEp) using the genes that code for the L-ASNases from *Erwinia chrysanthemy* (ErL-ASNase) and *Erwinia carotovora* (EcaL-ASNase) and screened using activity assays. A variant of the *E. carotovora* enzyme lacking detectable glutaminase activity was identified. Sequence analysis showed that this variant contained a single point mutation (Leu71Ile). Steady-state kinetic measurements and the analysis of the pH dependence of $V_{max}$ and $V_{max}/K_m$ of L-Asn hydrolysis showed that the mutation causes significant alterations in binding and catalytic properties. Analysis of the molecular model of the mutant enzyme showed that Ile71 may perturb the conformation of important amino acid residues in the linker region which directly affects the catalytic function. The Leu71Ile mutant enzyme was used to assemble a cuvette-based biosensor specific for L-Asn. The enzyme was immobilized by crosslinking with glutaraldehyde on the side of a transparent plastic cuvette. The sensing scheme was based on the colorimetric measurement of ammonia formation using the Nessler's reagent. Calibration curve was obtained for L-Asn, with useful concentration range of 0–100 μM for L-Asn. The method's reproducibility was in the order of ±2–5% and L-Asn recoveries were 102.1%.



## 1. Introduction

Interest in L-ASNase (EC 3.5.1.1) has grown considerably since this enzyme was found to have anti-tumour activity [1–5]. The bacterial L-ASNases from *Erwinia chrysanthemi* and *Escherichia coli* have been employed in the treatment of acute lymphoblastic leukemia (ALL) for almost 40 years [6–11]. The biochemical mechanism of asparaginase action in ALL therapy is simple: the transformed cells responsible for the disease have an absolute requirement for L-Asn due to a decreased or missing asparagine synthetase activity. Depletion of L-Asn in the blood, therefore, inhibits their proliferation and induces apoptosis [5,6,8].

L-ASNases (EC 3.5.1.1) catalyze the hydrolysis of L-Asn to produce L-Asp and ammonia, but are also able to hydrolyze, although less effective, L-Gln [12–18]. The enzyme is active as homotetramer [5,6]. The monomer consists of ~330 amino acid residues arranged in two domains: a large N-terminal domain and a smaller C-terminal domain [13,15,18]. The two domains are also connected by an approximately 20 residue flexible linker. 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. The flexible part of the active site regulates access to the binding pocket and provides the catalytic nucleophile Thr15, which is highly conserved for all L-ASNases [13,15,18].

One of the limiting factors of L-Asn treatment is the development of hypersensitivity, which ranges from mild allergic reactions to anaphylactic shock. The allergic reactions are normally treated by substitution with one of either the *E. chrysanthemi* or the *E. coli* enzymes, as they show no immunological cross-reactivity [9,10]. Additionally, L-ASNase therapy is often accompanied by toxic side effects, such as liver dysfunction and pancreatitis [11]. The enzymes from *Vibrio succinogenes* and guinea-pig serum lack hepatoxicity [12] and do not possess L-glutaminase activity, suggesting that this activity in the *E. chrysanthemi* and *E. coli* enzymes may be responsible for some of their toxic side effects. Glutamine is the major transport form of amino nitrogen in the blood and also an

*Abbreviations:* ALL, acute lymphoblastic leukemia; *Eca*L-ASNase, L-asparaginase from *Erwinia carotovora*; *Er*L-ASNase, L-asparaginase from *Erwinia chrysanthemi* 3937; Leu71Ile, mutant derived from StEP containing Ile at site 71; GDH, glutamate dehydrogenase; PAGE, polyacryamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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amino group donor for many biosynthetic reactions. A prolonged decline of plasma glutamine levels, therefore, impairs a variety of biochemical functions, especially those of the liver. Therefore, glutaminase-free L-ASNase is essentially required for successful clinical studies [13,14].

So far, only rational protein design techniques, such as site-directed mutagenesis, have been applied to engineer L-ASNases [19,20]. On the other hand, directed evolution has been proven effective in altering many key enzyme properties including stability, function in non-natural environments (such as organic solvents), product inhibition, expression in a recombinant host and substrate specificity, enantio-selectivity and/or regioselectivity [21,22]. Recently, our group achieved the development of a new engineered variant of *E. chrysanthemi* L-ASNse with high thermostability using *in vitro* directed evolution [23].

In the present study L-ASNase from *Erwinia carotovora* (*Eca*L-ASNase; [16]) and *E. chrysanthemi* (*Er*L-ASNase; [17]) were subjected to directed evolution, with a view to find an enzyme variant with low to negligible glutaminase activity. After one round of directed evolution one variant with a point mutation (Leu71Ile) was found. The experiments described in this paper represent the first experimental approach for altering the substrate specificity of this important enzyme using *in vitro* directed evolution approach. In addition, the Leu71Ile mutant enzyme was used to assemble a cuvette-based biosensor specific for L-Asn.

## 2. Materials

L-Asn and L-Gln were obtained from Serva (Heidelberg, Germany). α-Ketoglutaric acid and Sepharose CL6B from Sigma Co. (St. Louis, USA). NADH (disodium salt, grade II, ca. 98%) and crystalline bovine serum albumin (fraction V), were purchased from Boehringer Mannheim, (Germany). Nessler's reagent and glutamate dehydrogenase (GDH) were obtained from Fluka (Taufkirchen, Germany). All primers were synthesized and purified by MWG-biotech AG (Ebersberg, Germany). TOPO cloning kit and all other molecular biology reagents were from Invitrogen (USA).

## 3. Methods

### 3.1. Directed evolution of L-ASNase

Directed evolution of L-ASNase was carried out using the staggered extension process, (StEP, [24]) as described by Kotzia and Labrou [23]. Briefly, the plasmids (pCR®T7/CT-TOPO®) containing the nucleotide sequences of L-ASNase from *E. carotovora* (*Eca*L-ASNase; NCBI code AY560097) and *E. chrysanthemi* 3937 (*Er*L-ASNase; NCBI code AY560098) were used as parental sequences in the PCR reaction. The PCR reaction was carried out in a total volume of 50 μl containing 25 ng of each primer, 10 ng template plasmid DNA, 0.2 mM of each dNTP, 5 μl 10× *Taq* buffer, 1.5 mM $MgCl_2$ buffer and 2.5 units of *Taq* DNA polymerase (Stratagene, USA). The PCR procedure comprised 99 cycles of 30 s at 94 °C and 10 s at 46 °C.

### 3.2. Cloning, expression, and screening for low glutaminase activity mutants

Following completion of StEP, the resulting PCR amplicon was treated with the restriction enzyme *Dpn* I to eliminate parental plasmid DNA and was TOPO ligated to pCR®T7/CT-TOPO® expression vector. The resulting expression constructs pT7MutASNase were used to transform competent BL21(DE3)pLysS *E. coli* cells. *E. coli* cells, harbouring plasmids pT7MutASNase, were grown at 37 °C in 30 mL LB medium containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The synthesis of L-ASNases was induced by the addition of 1 mM IPTG when the absorbance at 600 nm was 0.6–0.8. Five hours after induction, cells were harvested by centrifugation at 8000 r.p.m. and 4 °C for 20 min, resuspended in potassium phosphate buffer (20 mM, pH 5.5), sonicated (for cell lysis), and centrifuged at 10,000 × *g* for 10 min. The supernatant was collected and assayed using L-Gln as substrate in order to identify promising mutants lacking glutaminase activity.

### 3.3. Purification of Leu71Ile mutant enzyme

The mutant enzyme Leu71Ile was purified on the affinity adsorbent L-Asn-Sepharose-CL6B [16]. Five hours after induction, cells were harvested by centrifugation at 8000 r.p.m. (4 °C) for 20 min, resuspended in potassium phosphate buffer (5 mM, pH 7.5), sonicated, and centrifuged at 10,000 × *g* for 20 min. The supernatant was collected and incubated at 4 °C under mild agitation for 1 h. Following incubation, the supernatant was again centrifuged at 10,000 × *g* for 5 min and the supernatant was applied to a column of L-Asn-Sepharose CL6B (2 mL, 1.5 cm × 1.5 cm I.D.) previously equilibrated with 5 mM potassium phosphate buffer, pH 7.5. Non-adsorbed protein was washed off with 20 mL equilibration buffer. Bound Leu71Ile was eluted with equilibration buffer containing 10 mM L-Asn. Collected fractions (1 mL) were assayed for L-ASNase activity and protein ($A_{280}$). The solution with purified enzyme was freed from L-Asn by dialysis (0.1 M potassium phosphate buffer including 0.1 mM EDTA and 1 mM β-mercaptoethanol, pH 7.2).

### 3.4. Assay of enzyme activity and protein

Enzyme assays were performed as described by Kotzia and Labrou [17]. Briefly, assays were performed at 37 °C at a Hitachi U-2000 double beam uv-vis spectrophotometer carrying a thermostated cell holder (10 mm pathlength). Activities were measured by determining the rate of ammonia formation, by coupling with glutamate dehydrogenase (GDH), according to Balcao et al. [25]. Alternatively, the rate of ammonia formation was measured at 37 °C using the Nessler's reagent [26].

Protein concentrations were determined at 25 °C by the method of Bradford [27] using bovine serum albumin (fraction V) as standard. Correction of protein concentration, due to small differences in purity between wild-type enzyme and the mutant enzyme, was achieved by using densitometric analysis of the SDS-PAGE gels [28].

### 3.5. Kinetic analysis

Steady-state kinetic measurements were performed in 0.1 M Tris–HCl buffer, pH 8.0 (or pH 8.2 for L-Gln), by varying the concentration of the substrate (L-Asn, L-Gln) [16,17,29]. The kinetic parameters $k_{cat}$ and $K_m$ were calculated by non-linear regression analysis of experimental steady-state data. Turnover numbers were calculated on the basis of one active site per subunit. Kinetic data were analyzed using the computer program GraFit (Erithacus Software Ltd.) [30].

### 3.6. pH Dependence of $V_{max}$ and $V_{max}/K_m$

The pH dependence of $V_{max}$ and $V_{max}/K_m$ of L-Asn hydrolysis was conducted in 50 mM Tris–50 mM acetic acid buffers. The steady-state kinetic parameters for Leu71Ile were determined by linear regression analysis of initial velocity data in the presence of variable concentrations of L-Asn at 37 °C. p$K_a$ values were estimated

by fitting the experimental data to the equation reported in [16], using the computer program GraFit (Erithacus Software Ltd.) [30].

### 3.7. Molecular modelling and computational analysis

The molecular modelling program WHAT IF [31] was used for predicting the conformation of the Leu71Ile mutant enzyme. The solved 3D structure of *E. carotovora* (PDB code 2jk0, [15]) was used to see the effect of the single amino acid change. The prediction of the conformation of the new side chains was performed as described by Chinea et al. [32]. Non bonded interactions were analysed by iMolTalk [33]. For inspection and analysis of protein models and crystal structures the program PyMOL was used [34].

### 3.8. Thermal stability of Leu71Ile mutant enzyme

Thermal inactivation of Leu71Ile mutant enzyme was monitored by activity measurements. Samples of the enzymes, in 10 mM $KH_2PO_4$ buffer pH 7, were incubated at 30–55 °C for 7.5 min. Subsequently the samples were examined for residual enzyme activity using activity assays. The half inactivation temperature ($T_{1/2}$) was determined from the plots of relative inactivation (%) versus temperature (°C). The half inactivation temperature ($T_{1/2}$) is the temperature at which 50% of the initial enzyme activity is lost after heat treatment.

### 3.9. Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli [35] on a slab gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Brilliant Blue R-250.

### 3.10. Development of cuvette-based biosensor for L-Asn

The Leu71Ile mutant enzyme was used to assemble a cuvette-based biosensor for L-Asn. The enzyme was immobilized by crosslinking with glutaraldehyde [36,37] as following: 3.0 mg of BSA were dissolved in 200 μl enzyme solution and the resultant solution was rapidly mixed with 5 μl glutaraldehyde solution (8% aqueous, grade I) and deposited as layer (0.5 mm) on the inner surface of the one side of a plastic cuvette (polystyrene). The enzyme–glutaraldehyde layer was allowed to stand at 4 °C for 45 min for crosslinking. The bioactive (enzymatic) layer (~0.2 enzyme units) was then rinsed repeatedly with 100 mM Tris/HCl buffer, pH 7.5, to remove any unreacted crosslinking agent and/or protein. The cuvette was filled with a solution of Tris/HCl buffer (100 mM, pH 7.5) and stored at 4 °C. L-Asn solutions (0–100 μM) were used to calibrate the biosensors at 25 °C. L-Asn and L-Gln recovery experiments were carried out in drinking water samples spiked with known amounts of L-Asn (2–200 μM) and L-Gln (100–2000 μM).

The cuvette-based biosensor was used for the determination of L-Asn in potato samples. Potato sample (50 g, obtained from the local market) were extracted (mortar and pestle) with water (100 mL) containing 2% (w/v) polyvinylpolypyrrolidone (PVPP) at 25 °C. To the extract 20 mL of 1 M perchloric acid was added. The extract was neutralised (KOH, 1 M) and centrifuged to separate insoluble material. An aliquot from the supernatant (100–200 μL) was used to analyse L-Asn levels using the biosensor. Validation of the results for L-Asn determination was carried out using the procedure described in a commercial kit (www.megazyme.com).

**Table 1**
Kinetic parameters of the wild-type and Leu71Ile mutant enzyme. Steady-state kinetic measurements were performed at 37 °C in 0.1 M Tris–HCl buffer, pH 8 (or pH 8.2 for L-Gln). 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 [30].

| Enzymes | Substrates | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) ($\times 10^3$) |
|---|---|---|---|
| Wild-type[a] | L-Asn | 0.085 ± 0.02 | 31.4 ± 1.44 |
| | L-Gln | 6.8 ± 1.32 | 0.46 ± 0.07 |
| Leu71Ile | L-Asn | 0.177 ± 0.028 | 1.234 ± 0.12 |
| | L-Gln | – | Not detected |

[a] Data for the wild-type enzyme were from [16] and were included for comparison.

## 4. Results and discussion

### 4.1. Identification and kinetic characterization of a mutant enzyme lacking glutaminase activity

In the present study, we employed *in vitro* directed evolution using the staggered extension process [24] as described by Kotzia and Labrou [23]. The two homologous L-ASNase sequences from *E. carotovora* and *E. chrysanthemi* (77% sequence identity) were used to provide the genetic diversity. The purified enzymes exhibit similar specific activity towards L-Asn as substrate, however, show significant difference in specific activity towards L-Gln [16,17]. For example, the glutaminase activity of *E. carotovora* enzyme is about 10% of that of the *E. chrysanthemi* enzyme. The library of gene variants generated was cloned and expressed in *E. coli*. Different clones from the mutant library were expressed in *E. coli* and assayed for glutaminase activity. One enzyme variant lacking glutaminase activity was identified and selected for further study. This enzyme variant was sequenced and found to be a single point mutant of the *E. carotovora* (*Eca*L-ASNase) enzyme. The mutation was at position 71 of the amino acid sequence (numbering according to mature enzyme sequence, PDB file 2jk0), with Leu being replaced by Ile (codon *TTA* → *ATA*). This mutation was presumably the result of an error introduced by the DNA polymerase.

Following sequencing, the mutant enzyme (Leu71Ile) was purified using affinity chromatography on immobilized L-Asn (L-Asn-Sepharose CL 6B) [16]. The mutant enzyme was purified 52.4-fold with 75.5% yield in a single step. The kinetic properties of the purified mutant Leu71Ile enzyme were investigated, and the $k_{cat}$ and $K_m$ parameters for L-Asn and L-Gln were determined by steady-state kinetic analysis (Table 1). The results showed that the mutant Leu71Ile no longer recognizes L-Gln as substrate even at high L-Gln concentration (1–50 mM). This is particularly important, since the glutaminase activity of the therapeutic preparations of L-ASNases has been implicated in causing side effects [12,14]. The $K_m$ value of the mutant enzyme for L-Asn was increased by 2-fold, compared to the wild-type enzyme. On the other hand, the $k_{cat}$ value of the mutant enzyme for L-Asn was significantly decreased (Table 1).

### 4.2. Structural basis of substrate specificity

To gain a deeper insight into the structural basis of substrate specificity, a molecular model of the mutant enzyme was constructed (Fig. 1). Analysis of the structure showed that Leu71 lies away from the active site (Fig. 1); in particular it lies at the α-helix α3 of the N-terminal domain. Ile71 forms the same van der Waals interactions compared with Leu71, however its side-chain was shifted towards Lys75 (2.68 Å), unlike Leu71 which leans close to Ser74 (2.71 Å).

The α-helix α3 (where Ile71 lies) is connected to the loop that interacts with the substrate. Since Leu71 is a conserved residue, it is possible that it plays an important role on the orientation of this

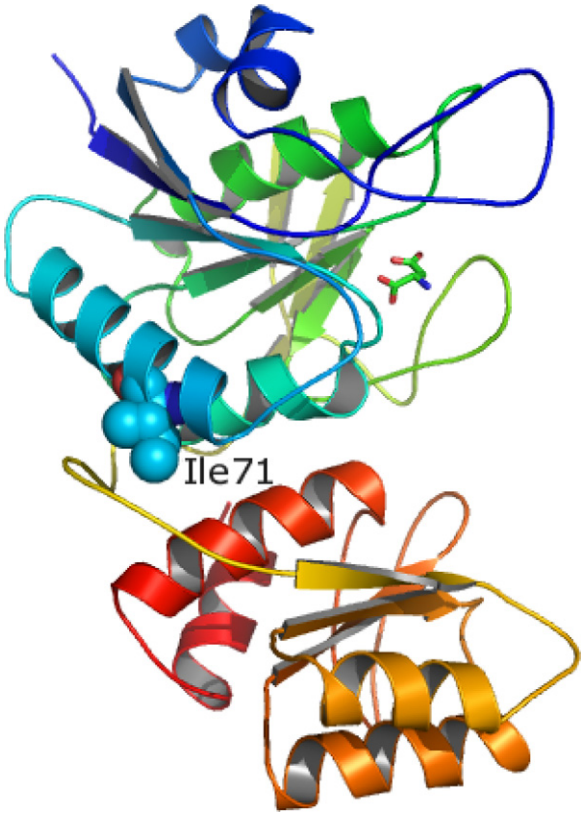


**Fig. 1.** Structural representations of the Leu71Ile mutant enzyme. Diagram of the modeled Leu71Ile mutant enzyme subunit. The mutated residue (Ile71) is shown in a space-fill representation and is labeled. L-Asp is shown in a stick representation. The model of the mutated enzyme was constructed using WHAT IF [31]. The figure was created using PYMOL [34].

loop. Presumably the mutation Leu → Ile changes the conformation of this loop leading to an altered binding of the substrate. In addition, the α-helix α3 is opposite to the linker that connects the two domains (N- and C-terminal) (Fig. 1). Analysis of the structures of the wild-type and Leu71Ile mutant enzyme showed that there are different interactions formed between Ile71 and the residues (e.g. Val211, Val214, Asp215 and Leu217) that lie in the linker compared with Leu71 (Fig. 2). Therefore, all these differences in the interactions of Ile71 with the residues Val211, Val214, Asp215 and Leu217 that lie in the linker of the two domains may lead to a redirection of the N-terminal domain of the mutant enzyme. Since the active site is located between the N- and C-terminal domains of two adjacent monomers, redirection of the N-terminal domain may affect substrate specificity in the Leu71Ile mutant enzyme. The results showed that amino acid changes distant from the active site can affect substrate specificity. Such changes can work by altering the conformation of active-site residues or the conformational dynamics of the entire protein, and are therefore difficult to anticipate.

### 4.3. pH Dependence of $V_{max}$ and $V_{max}/K_m$

The effect of pH on the $V_{max}$ and $V_{max}/K_m$ was investigated (Fig. 3) in an attempt to establish the involvement of specific functional groups of the enzyme and/or substrate in the catalytic reaction. Fig. 3A shows the $V_{max}$-pH profile for the L-Asn hydrolysis. The $V_{max}$-pH profile yields the p$K_a$, which reflects the ionization of the enzyme in complex with substrate [38]. Analysis of the pH-dependence of $V_{max}$ of the wild-type enzyme [16] suggests that the active site is comprised by two ionizable carboxylate groups, which have important roles in L-Asn binding and catalysis. We have shown for the wild-type enzyme that Asp115 and Glu82 undergo electrostatic interaction with the α-amino group of L-Asn and have been suggested to be involved in catalysis by playing an important role in the proper orientation of L-Asn [16].

In the case of Leu71Ile mutant enzyme (Fig. 3A) the $V_{max}$ decreased below pH 6 and appeared to be pH-independent in the pH range of 6.5–9.0. The pH transition observed seems to be controlled by a p$K_a$ of $4.95 \pm 0.05$, indicating that the protonation of a group in the acidic pH range is catalytically incompetent. This p$K_a$ may correspond either to the enzyme group or to the substrate group since there is also an ionizable group in the substrate. Compared with the wild-type enzyme (p$K_a$ 5.4) [16], the p$K_a$ of Leu71Ile mutant is shifted by 0.45 units towards the acidic pH range.

The $V_{max}/{K_m}^{Asn}$-pH profile for the mutant enzyme is shown in Fig. 3B. The profile shows two clear inflection points corresponding to p$K_a$ values of $5.5 \pm 0.06$ and $7.8 \pm 0.07$. The $V_{max}/{K_m}^{Asn}$-pH profile of the wild-type enzyme shows two p$K_a$ values of 5.4 and 8.5 [16]. These p$K_a$ values reflect the ionization of groups in the 'free' enzyme which are involved in substrate binding. Compared to the wild-type enzyme the p$K_a$ in the acid pH range remains constant whereas the alkaline inflection point is shifted by 0.75 units. These observations, in correlation with all other kinetic and structural results, suggest that the mutation Leu71Ile, even though is located at a position not readily supposed to affect substrate specificity, it has in fact perturbed the active site and therefore the substrate binding and the acid–base properties of the catalysis.

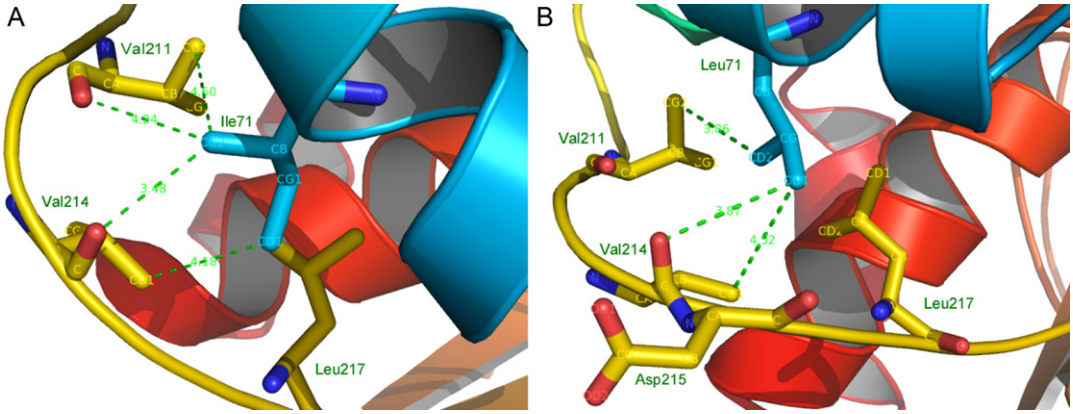


**Fig. 2.** Structural representations of the wild-type and Leu71Ile mutant enzymes showing interactions with residues that lie in the linker of the two domains. (A) Structural representation of the mutation site of the Leu71Ile mutant enzyme. (B) Structural representation of the mutation site of the wild-type *Eca*L-Asnase. Distances are shown as dashed lines and residues are labeled. The model of the mutated enzyme was constructed using WHAT IF [31]. All figure was created using PYMOL [34].

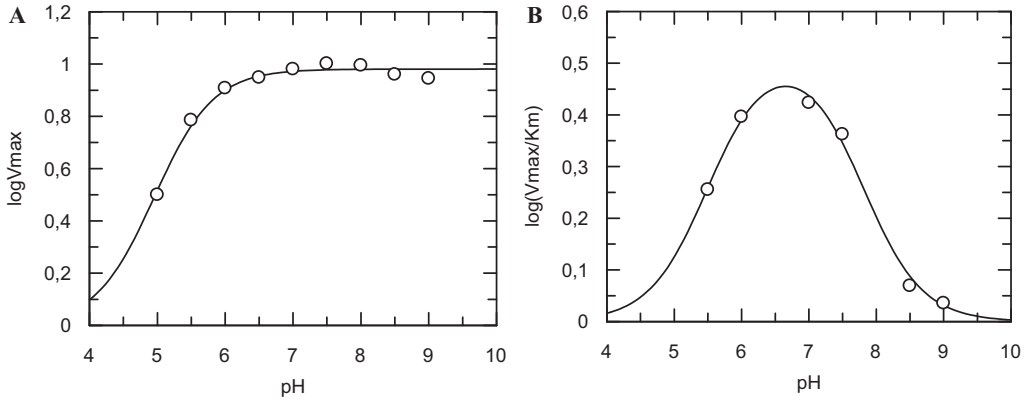


**Fig. 3.** pH-dependence of kinetic parameters of Leu71Ile for L-Asn hydrolysis (A and B). The kinetic parameters $V_{max}$ and $K_m$ were calculated by non-linear regression analysis of experimental steady-state data using the GraFit (Erithacus Software Ltd.) program [30]. (A) pH-dependence of log $V_{max}$ for L-Asn; (B) pH-dependence of log ($V_{max}/K_m$) for L-Asn.

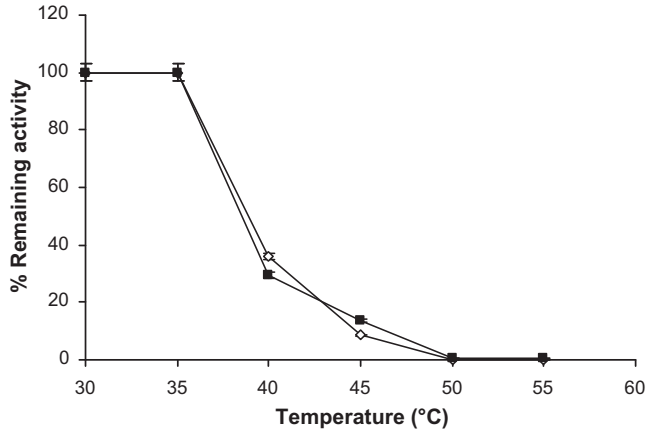


**Fig. 4.** Thermal inactivation curves. The residual activities of the wild-type (◊) and mutant Leu71Ile (■) enzymes were measured after heat treatment at various temperatures (30–55 °C) for 7.5 min.

### 4.4. Thermal stability of Leu71Ile mutant enzyme

To determine the structural stability of Leu71Ile mutant enzyme, its thermal stability was assessed by measuring its residual activity after heat treatment for 7.5 min at temperatures of 30–55 °C (Fig. 4). The half-inactivation temperature ($T_{1/2}$) of the mutant enzyme was found to be 38.2 ± 0.3 °C, which is almost identical (only 0.7 °C lower) to the wild-type [16]. Therefore the mutation does not affect the stability of the enzyme.

### 4.5. The basis of a cuvette-based biosensor for L-Asn

The high specificity towards L-Asn observed for the Leu71Ile mutant enzyme prompt us to investigate the development of a simple biosensor for the determination of L-Asn. This biosensor may be useful for the determination of L-asparagine in food or serum. Asparagine monitoring in human serum is necessary during leukemia therapy with L-asparaginase [39]. In food industry, it is now well known that when L-asparagine, ammonium ions, D-fructose and/or D-glucose are heated above approximately 160 °C, significant levels of the carcinogenic and neurotoxic compound acrylamide is formed, with obvious concerns for human health [40]. Foods like potato, rice, and corn, rich in both amino acids and carbohydrates, show alarming levels of acrylamide when fried or baked at high temperatures. The current understanding is that all food-borne acrylamide originates with free asparagine, along with minor contributions by other free amino acids. Therefore monitoring of L-Asn in raw food ingredients is of great interest [41].

In this sensor, the enzyme was immobilized by crosslinking with glutaraldehyde [37,42] and deposited as a layer on the inner surface of the one side of a plastic cuvette (Fig. 5). Glutaraldehyde is a bifunctional reagent that binds proteins through their free amino groups, thus forming a three-dimensional network. To stabilize this network mechanically and protect the enzyme activity, bovine serum albumin (BSA) was used. The activity of the bound enzyme was found to be 0.02 units/mg gel, whereas the nominal activity of the added enzyme was 0.04 units/mg gel. The immobilization process itself probably caused irreversible enzyme denaturation that led to a subsequent loss of enzyme activity (about 45%).

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. Calibration curve was obtained with useful concentration range of 0–100 μM and using the response obtained 30 min after addition of the substrates (Fig. 5). The reproducibility of sensor response to substrate, expressed by RSD, was in the order of ±2–5% ($N$ = 5). The application of the present method to the detection and determination of L-Asn was investigated by recovery experiments using water samples spiked with a known amount of L-Asn. L-Asn recoveries ranged between 96.2 and 115%, with an average value of 102.1% ($N$ = 5) (Table 2).

**Table 2**
L-Asn and L-Gln recovery experiments in drinking 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 | 2 | 2.3 | 115 | 100 | 0 | – |
| 2 | 50 | 48.1 | 96.2 | 500 | 0 | – |
| 3 | 100 | 105.1 | 105.1 | 1000 | 0 | – |
| 4 | 150 | 145.4 | 96.9 | 1500 | 0 | – |
| 5 | 200 | 194.4 | 97.2 | 2000 | 0 | – |

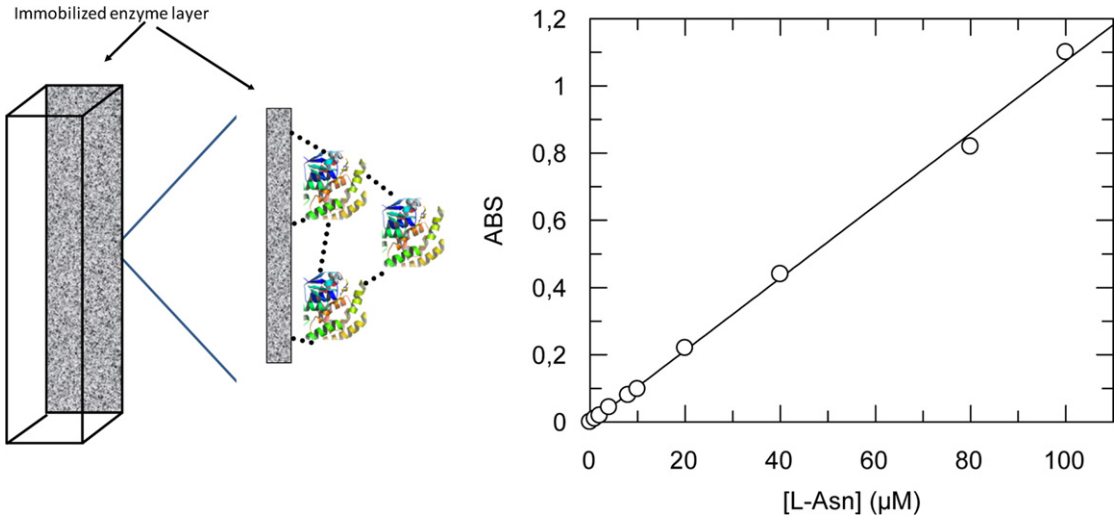


**Fig. 5.** (A) Experimental setup of the cuvette-based biosensor for L-Asn determination using immobilized L-ASNase. (B) Calibration graph obtained for L-Asn using the Leu71Ile mutant enzyme. Each point represents the average of three determinations.

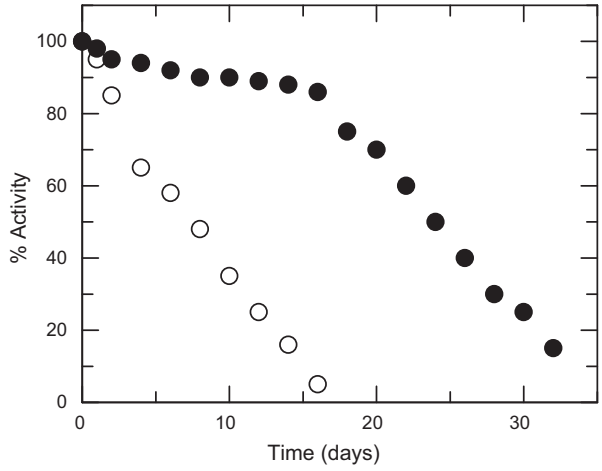


**Fig. 6.** Stability of the free (○) and immobilized Leu71Ile mutant enzyme (●) at 4 °C.

The cuvette-based biosensor was further used for the determination of L-Asn in potato samples. L-Asn levels in potato samples were determined between $920 \pm 48$ μg/g fresh weight. These are in agreement with the values for L-Asn in potato samples reported in the literature [43]. The results were further validated and showed very good reproducibility (error < 3–8%) using the procedure described in a commercial kit for L-Asn determination in food samples (www.megazyme.com).

So far three examples have been reported for the development of L-ASNase-based biosensors for the determination of L-Asn [39,44,45]. For example, L-ASNase from *E. coli* was immobilized on an ammonia gas sensor. The sensor exhibited a linear range between $2.0 \times 10^{-5}$ and $2.3 \times 10^{-3}$ M and was used for the determination of L-Asn in human serum [44]. In another example, the L-ASNase from the hyperthermophilic microorganism *Archaeoglobus fulgidus* was used. The enzyme was immobilized in front of an ammonium-selective electrode and used to develop a biosensor for L-Asn [45]. The biosensor had a detection limit of $6 \times 10^{-5}$ M for L-Asn. More recently, the L-ASNase from *E. coli* K-12 was co-immobilized with phenol red indicator [39]. Immobilization was carried out using nitrocellulose membrane, silicon gel and calcium alginate beads. The alginate bead system of immobilization was applied for the detection of L-Asn in normal and leukemia serum samples [39]. The main drawback of the reported methods was the use of wild-type enzymes with both asparaginase and glutaminase activity.

### 4.6. Stability of the immobilized enzyme

Fig. 6 shows the enzyme stability with time at 4 °C. It can be seen that the enzyme loses about 10% of its original activity within the first two weeks of use, whereas the free enzyme is completely inactivated. The immobilized enzyme activity decreases with time for the first two weeks in an almost linear manner. An average of 60 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 70% of its original value about three weeks after enzyme immobilization. These findings indicate that the mutant enzyme exhibits good stability upon storage at 4 °C which significantly improves the practical viability of this enzyme assay.

In conclusion, the results of the present work provide new data on the structural basis of the substrate specificity of L-ASNase that could not be directly predicted by its three-dimensional structure. The mutant enzyme display high specificity towards L-Asn and was used for the development of cuvette-based biosensor for L-Asn determination with good precision, accuracy, and short response time.

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