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

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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}) were determined for a number of substrates. 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%.

1. 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 Leukemia (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 [5,16–18]. 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

Abbreviations: BSA, bovine serum albumin; EcaL-ASNase, L-asparaginase from *Erwinia carotovora*; EcA, L-asparaginase from *Escherichia coli*; ErA, L-asparaginase from *Erwinia chrysantemi*; IPTG, isopropylβ-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.

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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 for 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* 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.

1.1. Materials

L-Asn, L-Asp and L-Gln were obtained from Serva (Heidelberg, Germany). Genomic DNA isolation kit, Nessler's reagent, Nα-Acetyl-L-Asn, succinamic acid and Nβ-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).

2. Methods

2.1. 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 24 h, 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 10x Pfu buffer and 2 units of Pfu DNA polymerase. The PCR procedure comprised 30 cycles of 2.5 min at 96 °C, 2 min at 46 °C and 3 min at 72 °C. A final extension time at 72 °C 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 °C 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 °C for 20 min, resuspended in sodium phosphate buffer (50 mM, pH 7), sonicated, and centrifuged at 10000*g* 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 CL6 B (2 mL, 1.5 × 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 Tris/HCl buffer and stored at −20 °C in glycerol/0.1 M Tris/HCl buffer pH 7.5, 50/50 (v/v).

2.2. 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.

2.3. 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, Nα-acetyl-L-Asn, succinamic acid, β-alanine amide). The kinetic parameters k_{cat} and K_m were calculated by non-linear regression analysis of the experimental steady-state data. Kinetic data were analyzed using the computer program GraFit (Erithacus Software Ltd., [26]).

2.4. 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].