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Optimization of glutaryl-7-aminocephalosporanic acid acylase expression in *E. coli*

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ABSTRACT

A recombinant glutaryl-7-aminocephalosporanic acid acylase (GLA) from *Pseudomonas* N176 has been over-expressed in BL21(DE3)pLysS *Escherichia coli* cells. By alternating screenings of medium components and simplified factorial experimental designs, an improved microbial process was set up at shake-flask level (and then scaled up to 2L-fermentors) giving a ~80- and 120-fold increase in specific and volumetric enzyme productivity, respectively. Under the best expression conditions, ~1380 U/g cell and 16,100 U/L of GLA were produced versus the ~18 U/g cell and the ~140 U/L obtained in the initial standard conditions. Osmotic stress caused by the addition of NaCl, low cell growth rate linked to high biomass yield in the properly-designed rich medium, optimization of the time and the amount of inducer's addition and decrease of temperature during recombinant protein production, represent the factors concurring to achieve the reported expression level. Notably, this expression level is significantly higher than any previously described production of GLAs. High volumetric production, cost reduction and the simple one-step chromatographic purification of the His-tagged recombinant enzyme, makes this GLA an economic tool to be used in the 7-ACA industrial production.

The industrial production of most cephalosporin derivatives is based on the chemical modification of 7-aminocephalosporanic acid (7-ACA)[1] as starting material. 7-ACA was initially obtained by the chemical deacylation of cephalosporin C (CephC) produced by fermentation of *Cephalosporium acremonium*. The chemical route requires toxic chemicals, involves cost-consuming steps and presents safety problems. Alternatively, the enzymatic conversion has long been explored to overcome these limits. Known processes of enzymatic transformation fall into two groups [1]: (i) the two-step process comprising the conversion of CephC into glutaryl-7-aminocephalosporanic acid (Gl-7-ACA), using the enzyme D-amino acid oxidase (DAAO, EC 1.4.3.3), and its subsequent hydrolysis to 7-ACA by a glutaryl-7-aminocephalosporanic acid acylase (GLA, EC 3.5.1.93); (ii) the one-step process conducted using a cephalosporin C acylase, i.e. a GLA that efficiently uses CephC as substrate.

Glutaryl-7-ACA acylases are members of the N-terminal hydrolases class of hydrolytic enzymes. GLAs have been identified in a variety of microorganisms mainly belonging to *Pseudomonas* and *Bacillus* genera. ORF of GLAs generally consists of a signal peptide, followed by a α-subunit, a spacer sequence (which is not present in the enzyme under investigation) and a β-subunit. The single, inactive, precursor polypeptide is post-translationally modified into a mature heterodimeric αβ enzyme by an auto-proteolytic cleavage upon folding, generating a new N-terminal residue: the proteolytic cleavage follows the translocation of the nascent protein into the periplasm and the activity is not acquired until the post-translational modification of the polypeptide is completed [2]. The active GLAs are $(\alpha\beta)_2$ heterotetramers that, according to substrate specificity and sequence conservation, have been divided into five classes. GLAs possess an activity to CephC that varies from 0 to 4% of the value determined on Gl-7-ACA [3,4]: members of class I (e.g., *Pseudomonas* sp. 130) and class II (e.g., *Pseudomonas* sp. N176) show the highest activity to CephC. Recently, improved variants of GLA possessing a 100-fold increased CephC/Gl-7-ACA catalytic efficiency and giving a significantly higher maximal activity to CephC (e.g., 3.8 U/mg protein at 25 °C) than to Gl-7-ACA, were obtained by a combined approach based on error-prone PCR mutagenesis, site-saturation mutagenesis and site-directed mutagenesis driven by a molecular modeling analysis [5].

Other factors conditioning biocatalytic applications of GLA are related to the quality of its preparations, which should be essentially free from contaminant enzymatic activities. Residual lactamases, esterases and acetylases may consume 7-ACA and thus reduce the product yield. Usually, recombinant GLAs are efficiently produced using engineered strains lacking of such activities and/

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[1] *Abbreviations used:* 7-ACA, 7-aminocephalosporanic acid; CephC, cephalosporin C; Gl-7-ACA, glutaryl-7-aminocephalosporanic acid; DAAO, D-amino acid oxidase; GLA, glutaryl-7-aminocephalosporanic acid acylase; LB, Luria–Bertani; LB Miller, Luria–Bertani Miller; SB, Super Broth; SBom, Super Broth NaCl-omitted; SB3, Super Broth 3; TB, Terrific Broth; TY, TYGPN broth.

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or by developing affinity chromatography strategies that allow single-step purification. In order to render the enzymatic process of 7-ACA competitive even from an economic point of view (the industrial level of GLA production is estimated $\geqslant$1500 millions of units per year), recent works have been focused on the optimization of recombinant GLA expression. Successful approaches comprise the improvement of the bioengineering process and strain development by conventional microbial genetics, as well as the use of recombinant DNA technology using different expression systems and hosts. In this work, we investigated the effect of the medium composition and of the fermentation conditions on the expression of a GLA with optimized properties for industrial application.

Materials and methods

Strain and growth conditions

Expression of the recombinant GLA protein was performed using BL21(DE3)pLysS *Escherichia coli* strain transformed with the pET24Δ-*GLA* plasmid as reported in [5]. Starter cultures were prepared growing a single colony of *E. coli* cells carrying the recombinant plasmid overnight at 37 °C in flasks containing LB broth, 30 μg/mL kanamycin and 34 μg/mL chloramphenicol. These cultures were diluted to a starting OD_{600nm} of 0.0025 in the production media containing the same antibiotics, and then incubated at 25 or 37 °C on a rotatory shaker at 200 rpm. The use of a very low inoculum proved essential to reduce cell lysis in the first phases of growth. The following liquid media were used: Luria–Bertani (LB, 10 g/L bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl, Sigma), Luria–Bertani Miller (LB Miller, 10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, Sigma), Super Broth (SB, 32 g/L bacto-tryptone, 20 g/L yeast extract and 5 g/L NaCl), Super Broth NaCl-omitted (SBom, 32 g/L bacto-tryptone and 20 g/L yeast extract), Super Broth 3 (SB3, 44 g/L bacto-tryptone, 30 g/L yeast extract and 10 g/L NaCl), Terrific Broth (TB, 12 g/L bacto-tryptone, 24 g/L yeast extract, 8 g/L glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4, Sigma) and TYGPN broth (TY, 20 g/L bacto-tryptone, 10 g/L yeast extract, 8 g/L glycerol, and 5 g/L Na_2HPO_4). All optimization studies were carried out in 500 mL baffled Erlenmeyer flasks containing 80 mL of liquid media.

Growth curves

Growth curves of recombinant *E. coli* BL21(DE3)pLysS cells containing the expression plasmid pET24Δ-*GLA* were investigated using LB Miller, TB, SB and TY media. Baffled (250 mL) Erlenmeyer flasks containing 50 mL of each medium were inoculated with the starter culture (initial OD_{600nm} = 0.1) and cells were grown at 37 °C with shaking (200 rpm). Both the optical density and the pH value were assayed every hour. The OD_{600nm} values were interpolated by using the *Gompertz* equation [6].

GLA expression, crude extract preparation and activity measurement

The enzyme expression was induced by addition of 0.6 mM IPTG at different times of growing. After induction, the cells were incubated overnight at 25 or 37 °C with shaking (200 rpm) and then harvested by centrifugation. Cell pellets were stored at −20 °C. Pellets were resuspended in lysis buffer (50 mM potassium phosphate, pH 7.5, 0.7 μg/mL pepstatin and 10 μg/mL DNaseI) and sonicated (4 cycles of 30 s each on ice). The insoluble fraction was removed by centrifugation at 39,000*g* for 1 h at 4 °C. Quantification of the total protein concentration in the crude extracts was performed by the biuret assay.

The activity assay was based on the hydrolysis of Gl-7-ACA to 7-ACA and the consequent formation of a yellow Shiff's base (with a maximum of absorbance at 415 nm) by the reaction of *p*-4-dimethylaminobenzaldehyde and the primary amino group of 7-ACA [5,7]. One unit of GLA is defined as the quantity of enzyme that converts 1 μmol of substrate per minute at 37 °C.

Factorial design

In order to optimize GLA production in *E. coli* three simplified Plackett–Burman factorial design experiments were performed [8]. The first experiment was designed to identify the effect of different concentrations of the SB medium components (bacto-tryptone, yeast extract and NaCl) on GLA expression. Three center points (internal controls) of the experiment were grown in standard SB medium and induced by 0.6 mM IPTG. The second and third factorial design experiments were then carried out to investigate the effect of NaCl (0–25 g/L range) and IPTG (0–1.2 mM range) as inducers using SB NaCl-omitted (SBom) or SB3 broths. The set up of the factorial design experiments and data analysis was performed using Statgraphics plus 4.1 software (Statistical Graphics Corp, Herndon, USA).

Production tests in 2-L bioreactor and GLA purification

Three different growths were carried out in 2-L working volume Applikon glass reactors. The starter culture was grown in LB medium and diluted up to an OD_{600nm} = 0.1 at the time of inoculation. Cells were grown in SB and SB3 broths at 37 °C, 600–900 rpm stirring and an aeration rate of 3 L/min, and induced with 0.6 mM IPTG or with 0.6 mM IPTG and 12.5 g/L NaCl. After the induction of protein expression (at OD_{600nm} = 4–5) the temperature was decreased to 25 °C: cells were harvested by centrifugation after 16–20 h from the induction.

Crude extracts were prepared by sonication (see above), adjusted to 1 M NaCl (final concentration), and loaded onto a HiTrap chelating affinity column (GE Healthcare Sciences) equilibrated with 50 mM sodium pyrophosphate buffer, pH 7.2, containing 1 M NaCl and 5% glycerol. The column was washed with this buffer until the absorbance value at 280 nm was that of the buffer. The bound protein was eluted with 20 mM sodium pyrophosphate buffer, pH 7.2, containing 500 mM imidazole and 10% glycerol. The fractions containing acylase activity were loaded on a PD10 Sephadex G25 column equilibrated with 20 mM potassium phosphate buffer, pH 8.0.

Results

Optimization of microbial growth and GLA expression

Under standard conditions, the recombinant GLA is expressed adding 0.6 mM IPTG at an OD_{600nm} = 0.8 (mid-exponential growth phase) to pET24Δ-*GLA* containing *E. coli* cells grown in LB Miller broth; cells were then incubated for additional 3 h at 25 °C. Under these conditions, a GLA specific activity of ∼0.28 U/mg proteins was obtained (corresponding to a volumetric productivity of ∼140 U/L fermentation broth). No activity was indeed detectable in recombinant *E. coli* cells transformed with pET24Δ empty vector.

In order to improve the productivity of the laboratory-scale microbial process for the production of the recombinant GLA, we first investigated the effect of different medium composition [9] both on the growth of BL21(DE3)pLysS *E. coli* cells carrying the pET24Δ-*GLA* plasmid at 37 °C and on their enzyme productivity. Growth data were analyzed using the *Gompertz* equation [6]: the observed growth rate (μm) is 0.98 h^{-1} in TB, 0.91 h^{-1} in LB Miller, 0.74 h^{-1} in TY and 0.69 h^{-1} in SB medium. The slowest growth rate and the highest biomass production is observed in SB medium, which gave 2-fold more biomass compared to LB Miller after 24 h