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Optimization of human D-amino acid oxidase expression in *Escherichia coli*

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ABSTRACT

Human D-amino acid oxidase (hDAAO) is a flavoprotein that plays a key role in the pathophysiology of schizophrenia. So far, the biochemical characterization of this enzyme has been hampered by the difficulty of expressing it in a common heterologous host such as *Escherichia coli*. Increasing amounts of recombinant hDAAO are indeed required for the investigation of its structure–function relationships and for the screening of new inhibitors to be used in the treatment of schizophrenia. A recombinant hDAAO has been over-expressed in BL21(DE3)Star *E. coli* cells. By alternating screenings of medium components at flask level and investigating physiological parameters in 2 L controlled batch fermentations, an improved, robust and scalable microbial process was set up giving almost a 40- and 4-fold improvement in volumetric productivity and specific activity, respectively. Under these conditions ~770 U/L culture hDAAO with a specific activity of ~0.4 U/mg protein and a specific productivity of 24.9 U/g biomass were produced. Optimization of medium ingredients, of the time and the amount of inducer's addition, pH control at the moment of induction and harvest, low mechanical shear stress regime during recombinant protein production, represent the factors concurring to achieve the reported expression level. Notably, this expression level is higher than any previously described production of hDAAOs. A yield of 100 mg of pure hDAAO/L culture thus became available in comparison to the 1–10 mg/L previously reported.

Introduction

This paper deals with the study of the human flavoprotein D-amino acid oxidase (EC 1.4.3.3, hDAAO). This peroxisomal enzyme contains noncovalently bound FAD and catalyzes the oxidative deamination of D-amino acids to their corresponding imino acids, whereas molecular oxygen undergoes reduction to hydrogen peroxide. In contrast to other known DAAOs, the human enzyme is a stable homodimer even in the apoprotein form and weakly binds the cofactor in the free form [1,2]. hDAAO exhibits optimal activity toward neutral D-amino acids and marginal activity toward basic ones, while acidic D-amino acids are oxidized by the flavoenzyme D-aspartate oxidase. Although the cDNA encoding hDAAO was isolated in 1988 [3], only recently the biochemical characterization of the human enzyme has been undertaken mainly because of its low expression level in a heterologous system. The physiological role of hDAAO in mammalians remained elusive until last decade [2]. In the brain, hDAAO degrades the transmitter D-serine, a potent activator of *N*-methyl-D-aspartate type glutamate receptors, and evidence suggests that hDAAO, together with its binding partner (inactivator) pLG72, plays a key role in the pathophysiology of schizophrenia [4,5]. Schizophrenia is a condition that affects up to 1% of the population; current therapies are inadequate and efforts are made to develop drugs to enhance *N*-methyl-D-aspartate type receptor function, e.g. by elevation of brain glycine and D-serine concentrations. Furthermore, administration of D-serine to schizophrenic patients treated with anti-psychotics has been reported to improve the positive and negative symptoms, as well as cognitive impairment [6].

Recent observations suggest a therapeutic strategy for schizophrenia based on the augmentation of brain D-serine levels by the hDAAO inhibition. As part of our effort to understand the *in vivo* modulation of the concentration of this neurotransmitter under physiological and pathological conditions and to develop selective hDAAO inhibitors, we hereby report on the development of an efficient and reproducible procedure for the over-expression of hDAAO in *Escherichia coli*.

Materials and methods

hDAAO cDNA subcloning

The cDNA encoding hDAAO (1041 bp encoding for a 347 amino acids long protein) was digested with NdeI and ligated into the

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similarly treated pET11b expression vector (Novagen) carrying a β-lactamase gene for ampicillin resistance [1]. Subcloning into pET24b vector (carrying a kanamicin resistance) was obtained from pET11b-hDAAO construct through digestion with NdeI restriction enzyme: the pET24b cloning vector was digested with NdeI and dephosphorylated with shrimp alkaline phosphatase. After ligation, the new construct was used to transform JM109 *E. coli* cells. Plasmid DNA was extracted with Nucleospin plasmid kit (Clontech) and digested with NdeI, NcoI/HindIII and NcoI/SmaI to check the correct insertion/orientation of hDAAO cDNA into the cloning vector.

Strain, media and growth conditions

Expression of the recombinant hDAAO protein was performed using BL21(DE3)Star *E. coli* strain and pET11b- or pET24b-hDAAO expression vectors. Preliminary trials showed that this host yielded the higher hDAAO expression as compared to BL21(DE3) and BL21(DE3)pLysS *E. coli* cells, which gave the 45% and 15% of its volumetric productivity/specific activity, respectively. Starter cultures were prepared growing a single colony of *E. coli* cells carrying the recombinant plasmid overnight at 37 °C in flasks containing LB (or TB) broth with 100 μg/mL ampicillin or 30 μg/mL kanamicin added. These cultures were diluted with the same media to a starting $OD_{600\ nm}$ of 0.1 and then incubated at 37 °C on a rotatory shaker at 200 rpm.

The following liquid media were used: Luria–Bertani (LB: 10 g/L bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl), Super Broth (SB: 32 g/L bacto-tryptone, 20 g/L yeast extract and 5 g/L NaCl), 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), Terrific Broth 5 (TB5: 12 g/L bacto-tryptone, 24 g/L yeast extract, 8 mL/L glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4, 5 g/L NaCl) and Tryptone Yeast (TY: 20 g/L bacto-tryptone, 10 g/L yeast extract, 8 mL/L glycerol, 5 g/L Na_2HPO_4). In some trials 0.5% (w/v) glucose was added. Experiments were carried out in 500 mL baffled Erlenmeyer flasks containing 80 mL of liquid media at 37 °C, unless otherwise specified, and 200 rpm. Both the optical density and the pH value were assayed every hour. Growth curves were generated by the interpolation of $OD_{600\ nm}$ values according to the *Gompertz* equation [7]. FAD, FMN, riboflavin and sodium benzoate (from Sigma–Aldrich) were added to the culture from concentrated solutions in water, filtered with a 0.22 μm membrane. In pH-controlled experiments, pH was maintained to the desired value by the addition of sterile NaOH and HCl solutions.

Expression of recombinant hDAAO

E. coli BL21(DE3)Star cells transformed with the above mentioned expression plasmids were grown in LB, SB, SB3, TB, TB5, or TY media to which were added 0.1, 0.3, 0.6, 0.9 or 1.2 mM IPTG at different phases of the growth curve. After induction, the cells were incubated for up to 24 h at 25 or 37 °C with shaking (200 rpm) and then harvested by centrifugation. Cell pellets were stored at −20 °C.

Alternatively, the Overnight Express™ Autoinduction System (Novagen®) [8] protocol was followed. In this case, cells were grown until the stationary phase (∼16 h) without IPTG addition in TB medium (System1, complex medium) or in a chemically defined medium (System2, defined medium containing induction solution, buffering solution, magnesium solution, metal mixture, amino acid mixture and methionine) before collection by centrifugation.

Crude extract preparation

Cell pellets were re-suspended in freshly prepared lysis buffer (4 mL per gram of wet cells) containing 50 mM sodium pyrophosphate, pH 8.3, 5% glycerol, 0.7 μg/mL pepstatin, 40 μM FAD, 5 mM 2-mercaptoethanol, 1.1 mM phenylmethanesulfonyl fluoride and 10 μg/mL DNaseI, and sonicated for five cycles of 30 s each, on ice in a Branson Sonifier 250. The insoluble fraction was removed by centrifugation at 39,000*g* for 1 h at 4 °C.

Activity assay

The DAAO enzymatic activity of crude extracts (50 μL) was determined measuring the oxygen consumption by a Hansatech oxygen electrode in 1 mL of 75 mM sodium pyrophosphate buffer pH 8.5, 56 mM D,L-alanine, and 0.2 mM FAD. The mixture was incubated at 25 °C with agitation and under conditions of air saturation [1,9]. One DAAO unit corresponds to the amount of enzyme that converts 1 μmol of substrate per minute. The specific activity of purified hDAAO is ∼8 U/mg protein [1]. Quantification of the total protein concentration in the crude extracts was performed by the biuret assay.

Scaling up to 2 L bioreactor

TB Medium was used as production medium in 2 L working volume P-100 Applikon glass reactor (height 25 cm, diameter 13 cm) equipped with a AD1030 Biocontroller and AD1032 motor. Cultivations in fermentor were carried out at 37 °C, 500–1000 rpm stirring (corresponding to 1.17–2.35 m/s of tip speed) and 2 L/min aeration rate. Foam production was controlled by the addition of Hodag antifoam through an antifoam sensor. In some runs, pH was controlled by the addition of 0.47 M H_2SO_4 or 0.625 N NaOH and dissolved oxygen concentration was maintained at 30% set point by stirring cascade control. The starter culture was grown overnight in LB or TB medium and diluted up to an initial $OD_{600\ nm}$ of 0.1, unless otherwise stated. After IPTG addition, cells were maintained under the mentioned conditions for further 16 h and harvested by centrifugation.

Results

Optimization of microbial growth and hDAAO expression

Initially, the recombinant hDAAO was expressed adding 0.6 mM IPTG at an $OD_{600\ nm}$ of 0.8–1 (mid-exponential growth phase) to *E. coli* cells transformed with pET11b-hDAAO plasmid and growing in LB broth at 37 °C; cells were then incubated for an additional 16 h at 37 °C. Under these conditions, a hDAAO specific activity of $\leqslant$0.1 U/mg protein was obtained, corresponding to a volumetric productivity of ∼19 U/L culture and to a specific productivity of 3.3 U/g biomass [1]. No activity was detectable in recombinant *E. coli* cells transformed with pET11b empty vector. Most of the following experiments had also been performed by using BL21(DE3)Star *E. coli* cells containing pET24b-hDAAO expression vector instead of pET11b-hDAAO, to check whether the plasmid stability in the recombinant host may affect expression results. Indeed, no difference in hDAAO productivity and activity was revealed between the two transformed recombinant strains, whose selection is based on the aminoglycoside kanamycin (pET24b) or the β-lactam ampicillin (pET11b).

In order to improve the productivity of the laboratory-scale microbial process for the recombinant hDAAO, we first investigated the effect of medium composition [10] both on the growth of BL21(DE3)Star *E. coli* cells carrying the pET11b-hDAAO at 37 °C