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

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A B S T R A C T

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 concerning 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.

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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 mammalian 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
similarly treated pET11b expression vector (Novagen) carrying a β-lactamase gene for ampicillin resistance [1]. Subcloning into pET24b vector (carrying a kanamycin 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, Ncol/HindIII and Ncol/Smal 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)pLYS S 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 kanamycin 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_{2}PO_{4} and 72 mM K_{2}HPO_{4}), Terrific Broth 5 (TB5: 12 g/L bacto-tryptone, 24 g/L yeast extract, 8 g/L glycerol, 17 mM KH_{2}PO_{4} and 72 mM K_{2}HPO_{4}, 5 g/L NaCl) and Tryp- tose Yeast (TY: 20 g/L bacto-tryptone, 10 g/L yeast extract, 8 mL/L glycerol, 5 g/L Na_{2}HPO_{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 biuret assay.

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 20 ml Apikon 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_{2}SO_{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 <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 amino glycoside 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

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 μg/mL FAD, 5 mM 2-mercaptoethanol, 1.1 mM phenylmethanesulfonyl fluoride and 10 μg/mL DNasel, 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.
and on their enzyme productivity. Growth data in six different media containing various combinations of carbon (glycerol) and nitrogen sources (yeast extract and bacto-tryptone), NaCl and phosphate buffer were analyzed using the Gompertz equation [7]: the observed specific growth rates were 1/h in LB, 0.98/h in SB, 1/h in TB, 0.97/h in TB5, 0.94/h in SB3 and 0.72/h in TY medium. Using SB, TB, TY, SB3 and TB5 media, higher biomass level was reached in comparison to LB, with duplication times variable between 41 and 46 min (57 min for TY medium) (Fig. 1A). Highest biomass production of 19.4 g/L and 37.6 g/L were achieved in SB and TB media respectively, in comparison to the 5.9 g/L obtained in LB.

Beside higher biomass production, TB medium also sustained the higher expression of hDAAO, measured as volumetric productivity. Growing the cells in TB medium for an additional 16 h at 37 °C after the induction with 0.6 mM IPTG in the middle-exponential phase, gave up to a 20-fold increase, in comparison to the enzyme produced under standard conditions (Fig. 1B). The specific productivity (U/g cell) was about doubled in TB versus LB medium, even if the specific activity in the crude extract remained below the level of 0.1 U/mg protein. It is worthy of mention that SB3 medium containing 10 g/L of NaCl, which was previously optimized in our lab for the production of glutaryl-7-aminocephalosporanic acid acylase in E. coli [10], supported a lower hDAAO expression than TB medium. Similarly, addition of NaCl to TB medium (TB5) did not give any improvement (Fig. 1B). In all the conditions so far tested, decreasing from 37 °C to 25 °C the growth temperature after induction, caused a 30% reduction in the level of hDAAO productivity (data not shown), as previously indicated by other authors on microbial-sourced DAAOs [11].

In order to compare our data on hDAAO expression with those reported by other authors [12], a variant of classical TB medium containing 0.5% (w/v) glucose was also tested. The pH of this medium dropped down to ~5.0 after 8 h from inoculation and the hDAAO volumetric productivity was reduced to the 10% of the value achieved in control TB. Batch-addition of glucose to recombinant E. coli cultivation is known to enhance acetic acid formation: relatively low concentrations of acetic acid are inhibitory to protein production first, and then retard E. coli growth [13]. Moreover, residual presence of glucose at the moment of IPTG induction may exert inhibitory action due to the catabolite repression of lac promoter [8,14].

Optimization of induction

Since E. coli transformed cells grew with a different kinetics and reached variable level of biomass production depending on medium composition, hDAAO expression was induced at different growth stages (OD600 nm of 0.8, 2 and >11) with 0.6 mM IPTG and harvested at different times after induction (2, 4 and 24 h). Results, reported in Fig. 2 as volumetric productivity (U/L) and specific activity in the crude extract (U/mg protein), confirm that the best level of hDAAO expression was achieved in TB medium, inducing the cells in stationary phase (OD600 nm ~16) after 16 h from the inoculum, and collecting the cells 4 h after IPTG addition. Both SB and TY media yielded lower hDAAO expression levels with a slightly different kinetics (best yield was obtained with induction at OD600 nm ~2.0 and harvest after 4 h). Specific activity ranged from 0.01 to 0.1 U/mg protein (Fig. 2).

In the best conditions so far achieved (TB medium, induction at OD600 nm of 16.7 and harvest after 4 h from IPTG addition: conditions whose volumetric and specific productivity is set to 100% in the following graphics), the effect of a range of IPTG concentrations from 0.1 to 1.2 mM was investigated. Fig. 3 shows that an increase in hDAAO volumetric productivity was associated to a decrease in IPTG addition, with a 1.8-fold improvement at 0.1 mM inducer concentration. This result represents a practical advantage, since IPTG is the mostly expensive component in E. coli recombinant protein production. The level of protein production observed in the absence of IPTG (~20% of control, Fig. 3) may not be neglected. It is worthy to note that in the trials with glucose added to TB (see above), this basal level was also reduced to 10%, indicating that the inhibitory effect is more likely a consequence of the environment acidic shift than depending on the block of IPTG action by catabolite repression.

The differential role of glucose and lactose in modulating the lac promoter has been exploited in the so-called Overnight Expression System [8,15], which is based on the use of two different media -TB as a complex one (System1) and a chemically defined medium (System2), whose composition is not completely unveiled- for the production of recombinant protein in E. coli without the need of IPTG addition. Recombinant proteins such as transglutaminase from Bacillus subtilis [16], have been successfully produced in E. coli following this method. When the two auto-induction systems were used with our pET11b-hDAAO containing BL21(DE3)S-tar E. coli cells, in System1 the volumetric productivity fell down to nearly 50% of what measured in our best conditions, whereas in System2 the level of hDAAO expression was comparable to the basal one, usually detected in the absence of IPTG induction (see above and Fig. 3).

Another approach tempted to enhance hDAAO production was the addition of putative enzyme stabilizing agents such as the cofactor FAD and its precursors FMN and riboflavin, or the competitive inhibitor sodium benzoate [1]. Cofactors and sodium benzoate...
were added at 0.1 mM concentration at the moment of IPTG supplementation: in these conditions a slight decrease of the enzyme productivity was observed in comparison to the control (Fig. 4).

**Fermentor scaling up and process definition**

In order to verify scaling-up feasibility of the flask conditions for hDAAO expression to larger volumes, batch fermentations were run at 2 L fermentor scale. Fermentation studies in bioreactor included experiments with different regimes of mechanical stirring, dissolved oxygen concentration (pO$_2$) and pH control. Impact of inoculum source [17] and volume was taken into account, too. Fig. 5A shows the typical profile of the cultivations carried out in TB medium added with 0.1 mM IPTG after 7.5 h from inoculum and harvested after additional 16 h. After a lag phase of 4 h, the culture started to grow exponentially till 10 h after inoculum: in this latter phase there was a rapid consumption of dissolved oxygen. The system was programmed in a way that when pO$_2$ fell

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**Fig. 2.** Kinetics of hDAAO production in TB, SB and TY media: volumetric productivity (U/L, left panels) and specific activity (U/mg protein, right panels) obtained inducing protein expression at different growth phases (OD$_{600}$ nm, z-axis) and harvesting the cells at different times after IPTG addition (2, 4 and 24 h, x-axis).
down below 30% of saturation, the mechanical stirring was increased from 500 to 1000 rpm (from 1.17 to 2.35 m/s tip speed) to allow a better oxygen distribution and avoid prolonged anaerobiosis. The pH remained at the initial value of 6.7 for 24 h after the inoculum, then it suddenly dropped down to values around 6.2, most likely due to the production of acetic acid in the phase of active growth and dramatic oxygen consumption. After a reproducible second spike between 6 and 8 h of growth, pH increased to values around 7.6 or higher due to incoming cell lysis at the stationary phase of growth. Higher final pH values were observed in those runs where higher tip speeds were needed to compensate oxygen limitation (data not shown). Interpolation of OD600 nm measurements along the growth curve with the Gompertz equation indicated that higher biomass production (48.5 g/L, corresponding to a final OD600 nm of 21) and higher specific growth rate (0.79/h) could be measured at fermentor scale in comparison to the flask level. Under these experimental conditions, a volumetric productivity of 243 U/L, comparable to the one achieved at flask level, was obtained. A specific productivity of 5 U/g cell and a specific activity of 0.062 U/mg protein showed that a slightly less hDAAO was produced in fermentor than in flask, but this was compensated by the increased final amount of biomass.

Interestingly, starting the inoculum from a daily fresh subcloned single colony instead that from a glycerol stock of pET11b-hDAAO containing BL21(DE3)Star E. coli cells, yielded a better reproducibility and a mean increase of 36% in the final volumetric hDAAO productivity. Furthermore, a slightly higher (~20%) hDAAO specific activity in the crude extract was obtained starting the cultivation from an optical density (OD600 nm of 0.05) lower than the initial condition so far used (OD600 nm of 0.1).

Effect of pH on expression level

The observation of a peculiar pH trend in fermentor’s cultivations (Fig. 5A), suggested that we investigate the effect of pH values at the time of inoculum, induction and harvest. These studies were conducted in two ways. Process was scaled down to shaken flasks, whose pH at the time of inoculum, induction and harvest was artificially set to a desired value. It was previously reported for microbial-sourced DAAOs that the best initial pH was 7.5 and that the cell growth and enzyme activity values decreased when the initial pH was lower than 7.0[11]. Our results show that a crucial parameter to predict hDAAO productivity is indeed represented by the pH at the time of induction: as shown in Fig. 6, the best results in hDAAO expression were achieved when the pH at induction was kept at around 6.7. Optimal pH at the harvesting time was around 7.1, confirming that higher pH values (around 8), which usually indicate cell lysis and proteolytic activity, are not recommended for protein production in E. coli.

Final optimization

New experiments were performed in a 2 L fermentor using a more diluted inoculum from a newly re-isolated colony, controlling the pH from the moment of induction and reducing as much as possible the mechanical shear stress of the cells. The system was maintained in “mild condition” of stirring (500 rpm in lag phase and after induction, 700 rpm during the exponential phase of growth) during the entire fermentation. As shown in Fig. 5B, the lag phase was reduced and the exponential phase of growth started after about one hour from the inoculum. Under these conditions pO2 dropped to 0% after 4 h from the inoculum. Cultures
experiencing prolonged oxygen limitation did not reach the growth parameters observed either at flask level or in the first fermentation trials: 29.7 g/L of cells (corresponding to an OD_{600} of 10.1 at the end of cultivation) were obtained with a specific growth rate of 1.27/h. The pH profile (Fig. 5B) was similar to the one previously observed (Fig. 5A), even if its value was controlled at pH 6.7 at the moment of IPTG addition and then kept below 7.4 until the harvest. These slower growing cultures limited in oxygen indeed expressed up to 770 U/L of hDAAO with a specific activity of 0.41 U/mg protein and a specific productivity of 24.9 U/g cell. The process was reproducible since it gave similar results (±20% in volumetric productivity) in three different replicates. These values are definitively higher than those reported in the literature for hDAAO production in E. coli cells [11,12,18,19] and represent almost a 40- and 4-fold improvement in volumetric productivity and specific activity, respectively, in comparison to the standard initial conditions.

Discussion

Characterization of human DAAO, which is required for a full understanding of its physiological role, has been hampered by the difficulty of expressing it in a common heterologous host such as E. coli. With an estimated yield of 1 mg enzyme/L culture, it has been proved to be a difficult protein to express in recombinant form, making isolation of the pure enzyme a challenging task [19]. More recently an initial work on medium composition and purification procedure has led up to 5 mg purified enzyme/L culture (95% homogenous) with an overall purification yield of 55% [12]. In this case the cultivation of E. coli BL21(DE3) strain containing the pET11b-hDAAO expression vector was carried out in a modified version of the classical TB medium lacking phosphate buffer, but with the addition of 0.5% (v/v) glucose. Cells were induced with 0.1 mM IPTG and harvested after 24 h for protein purification. In our preliminary trials, the same host-vector expression system was induced with 0.6 mM IPTG during the exponential phase of growth in LB medium and the cells harvested after overnight growth at 37 °C [1]. The best yield so far achieved was of 6.7 mg of hDAAO/L culture, and the process was highly irreproducible. Moreover, a basal level of expression (corresponding to <2 mg of hDAAO/L culture) was often observed. Other authors [18] reported that hDAAO expression was improved by increasing the amount of IPTG (up to 1 mM) and by adding the inducer at OD_{600} ~2.0: up to 11.5 g of cell/L culture and ~24 mg hDAAO/L culture were achieved in these last conditions It is likely that even this protocol suffered from low reproducibility, since the same authors most recently decided to re-adopt previous conditions, which yielded only 5 mg hDAAO/L culture [20].

Hereby we report a systematic investigation on the effect of medium components and process parameters on hDAAO expression in E. coli. The aim was not only to achieve an increasing yield of the recombinant expressed protein, but also to design a robust and reliable process at lab bioreactor scale, which can be eventually scaled up to major volumes. For these reasons, our approach to improve a stable hDAAO expression protocol in E. coli was first based on the screening of various medium components, induction conditions, antibiotic selection markers and analysis of growth and expression kinetics at flask level. From this phase, a better growth supporting medium (TB) was selected, and optimized (and less expensive) conditions for protein induction by IPTG addition were defined. The resulting final improvement of about 12-fold in volumetric productivity was not accompanied by a significant increase in the specific activity. The second phase, essentially performed in bioreactors, allowed a better comprehension of physiological parameters in hDAAO producing cells. E. coli is an anaerobic facultative microorganism, whose metabolism may shift from respiratory to fermentative, producing in the latter case a mixture of acids, including the auto-toxic acetic acid. It is generally recognized that high density cultures of E. coli are limited by the oxygen availability in normally aerated fermentors and in that conditions the production of acetic acid inhibits heterologous protein production [13]. In our case, the production of hDAAO seemed more affected by the cell shear stress due to the mechanical stirring, which is required to better oxygenize the cultures, rather than by the prolonged oxygen limited growth phase. In some added-value protein productions, pure oxygen has been supplied to support intensive growth of E. coli producing recombinant proteins, but this practice is extremely costly, risky and it cannot be easily scaled-up in fermentation plants [21]. pH Values lower than neutrality seemed to favor the efficacy of IPTG induction of protein expression, whereas, as reported in many other cases [21], early entry into the stationary phase and consequent cell lysis may be avoided (due to the high proteolytic activity leading to protein degradation). Anyway, an indirect effect of oxygen limitation in slowing down the endogenous protein production in favor of the heterologous expression may not be ruled out.

In conclusion, a 40-fold improvement in volumetric productivity accompanied by a 4-fold increase in the specific activity was achieved in a reproducible way by the optimization of fermentation parameters. About 100 mg hDAAO/L culture thus became available and the process could be scaled up to 50 or 200 L working volume fermentors allowing production of grams of hDAAO. This huge amount of recombinant hDAAO is required at the lab-scale for the investigation of the structure-function relationships of a flavoenzyme involved in brain D-serine metabolism [2,5] and for the screening of new DAAO inhibitors to be used in the treatment of schizophrenia [22-24].

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