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High-level expression of *Trigonopsis variabilis* D-amino acid oxidase in *Escherichia coli* using lactose as inducer

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Abstract

The use of lactose as inducer for the expression of *Trigonopsis variabilis* D-amino acid oxidase gene (*daao*) was investigated in *Escherichia coli* regulated by T7 or T5 promoter. The *daao* gene was prepared by reverse transcriptase-polymerase chain reaction and cloned into pET21b and pQE-30 to yield pET-DAAO and pQE-DAAO, respectively. The His_6-tagged DAAO was expressed in *E. coli* and had a M_r value of approximately 39.3 kDa. In lactose-induced *E. coli* BL21 (DE3) (pET-DAAO), the expressed DAAO could comprise up to 15% of total soluble proteins and a productivity of 23.4 U ml^{-1} was obtained.

Introduction

D-Amino acid oxidase (DAAO, EC 1.4.3.3) stereospecifically catalyzes the oxidative reaction of the amino group of D-amino acid to produce its related keto acid and H_2O_2. It has several uses in chemical and pharmaceutical industry, such as the production of L-amino acids (Fischer *et al*. 1995) and antibiotics (Serizawa *et al*. 1980). The most important use of it is in a two-step process for converting cephalosporin C into 7-aminocephalosporanic acid (7ACA), a key intermediate for the synthesis of cephem antibiotics (Chen *et al*. 1991).

The expression of pig kidney (Setoyama *et al.* 1996), *Fusarium solami* (Isogai *et al.* 1990), *Rhodotorula gracilis* (Molla *et al.* 1998), and *Trigonopsis variabilis* (Alonso *et al.* 1999) DAAO proteins in *Escherichia coli* have been reported. Interestingly, the expressed pig kidney DAAO could reach 49% of the total soluble protein; however, the low specific activity renders the recombinant enzyme unsuitable for industrial uses (Setoyama *et al.* 1996). Using *E. coli* T7 expression system, a fermentation yield of 23 U ml^{-1} is achieved for the production of recombinant chimeric *R. gracilis* DAAO (Molla *et al.* 1998). Recently, the *T. variabilis* DAAO has been engineered to facilitate its overproduction in *E. coli* and downstream processing, while the *daao* gene is not efficiently expressed in the host cell and most of the enzyme is produced in its apoenzyme form (Alonso *et al.* 1999). In this study, we describe the lactose-induced expression of active *T. variabilis* DAAO by an *E. coli* expression system.

Materials and methods

Strains, vectors, and growth conditions

Trigonopsis variabilis CCRC 21509 was obtained from the Culture Collection and Research Center of FIRDI (Hsinchu, Taiwan). *E. coli* DH5α (Hanahan 1983) was used for the preparation and construction of plasmids. The overproduction of DAAO protein was carried out in *E. coli* BL21(DE3) (Studier & Moffatt 1986). Plasmids used were pQE30 (Qiagene Inc., CA, USA) and pET21b (Novagene Inc., WI, USA). *E. coli* was grown aerobically in Luria-Bertani medium (LB). As required, LB was supplemented with 100 μg ampicillin ml^{-1}. The yeast was grown in 50 ml YCBA

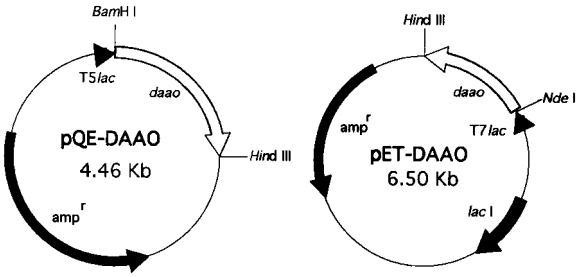

Fig. 1. Schematic diagram of the expression plasmids.

medium (0.67% Yeast Carbon Base and 0.2% D,L-alanine) at 30 °C for 72 h. Cells were inoculated to 1000 ml YCBDA medium (0.65% Yeast Carbon Base and 0.2% D-alanine) and grown for another 24 h, and then harvested for total RNA isolation.

Molecular techniques

Restriction enzymes and T4 DNA ligase were purchased from New England Biolab (MA, USA) and used according to the manufacturer's instructions. Standard protocols were used for manipulation of DNA and transformation of *E. coli* strains (Sambrook *et al.* 1989). Total RNA of *T. variabilis* was isolated by the procedure described by Chirgwin *et al.* (1979). Primers used were designed according to the nucleotide sequence of *daao* gene (Komatsu *et al.* 1987). The PCR reaction mixture, in 100 μl, contained 1 ng template DNA, 0.2 mM each dNTP, 200 pmole each primer, 1× PCR buffer and 5 U *Taq* polymerase. The PCR reaction was performed in a GeneAmp PCR system 2400 (Perkin Elmer, CO, USA) for 30 cycles under the following condition: 94 °C for 1.5 min, 65 °C for 2 min, and 74 °C for 2 min, followed by a final extension at 74 °C for 10 min. Sequence determination was performed with a Sequenase DNA sequencing kit (United States Biochemical, OH, USA).

Construction of expression plasmids

A SuperScript Preamplification System (BRL, MD, USA) and *Taq* DNA polymerase (BRL, MD, USA) were used to perform reverse transcriptase-polymerase chain reaction (RT-PCR). The first strand cDNA was synthesized from total RNA by the Preamplification System. A 5′ primer Trv1 (5′-ACAGCATATGGCTAAAATCGTTG-3′), incorporated with a *Nde*I site, and a 3′ primer Trv2 (5′-CATAAGCTTAGAAGGTTTGGACGAG-3′), incorporated with a *Hin*dIII site, were used to amplify *daao* gene from the first strand cDNA. The resulting DNA fragment was purified by agarose gel electrophoresis and recovered by GeneClean II kit (Bio 101, CA, USA). The recovered DNA fragment was then cloned into plasmid pET21b between *Nde*I site and *Hin*dIII sites to yield pET-DAAO (Figure 1), in which the expression of *daao* gene is under the control of T7 promoter. Another expression plasmid, pQE-DAAO (Figure 1), was also constructed by inserting the DNA fragment amplified by primers Trv3 (5′-GCTGGATCCATGGCTAAAATCGTTGTTATTGG-3′) and Trv2 into plasmid pQE30 between *Bam*HI and *Hin*dIII sites. In pQE-DAAO, the expression of *daao* gene is under the control of T5 promoter.

Expression of DAAO enzyme

For evaluation of DAAO production, *E. coli* DH5α carrying pQE-DAAO and *E. coli* BL21 (DE3) harboring pET-DAAO were cultivated at 37 °C in 100 ml LB medium. When the optical density at 600 nm reached 0.8, the cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at 0.4 mM for 3 h or with lactose at 2 mM for 7 h. Cells were then collected by centrifugation and resuspended in 3 ml of 100 mM phosphate buffer (pH 7.0). The cell suspensions were disrupted by sonication on ice (30-s bursts for 3 min). The extracts were clarified by centrifugation at 10 000 × g for 5 min at 4 °C and the resulting materials containing soluble DAAO were used for enzyme assay. Proteins were resolved on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie Blue.

Enzyme assay

DAAO activity was assayed by measuring the production of keto acid according to the Freidemann method described previously (Lee *et al.* 1994). The reaction mixture contained 100 mM D-alanine, 100 mM potassium phosphate buffer (pH 8.0), 400 U of bovine liver catalase, and an appropriate amount of crude extracts in a final volume of 1 ml. One unit (U) of DAAO activity was defined as the amount of the enzyme for producing 1 μmol pyruvate per min at 37 °C. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as standard. Specific activity is expressed as units per mg protein. Productivity is expressed as units per liter of medium.

Table 1. Production of D-amino acid oxidase by *E. coli* transformants.

Plasmid	Specific activity (U mg^{-1} protein)			Productivity (U ml^{-1})		
	No inducer	IPTG	Lactose	No inducer	IPTG	Lactose
pET-21b	ND[a]	ND	ND	ND	ND	ND
pQE-30	ND	ND	ND	ND	ND	ND
pET-DAAO	ND	13.5 ± 0.4[b]	20.7 ± 1.3	ND	10.3 ± 1.8	23.4 ± 4.7
pQE-DAAO	<0.2	4.8 ± 0.6	0.4 ± 0.1	<0.1	2.8 ± 0.9	1.5 ± 0.3

[a]ND, not detected.
[b]Standard deviation ($n = 3$).

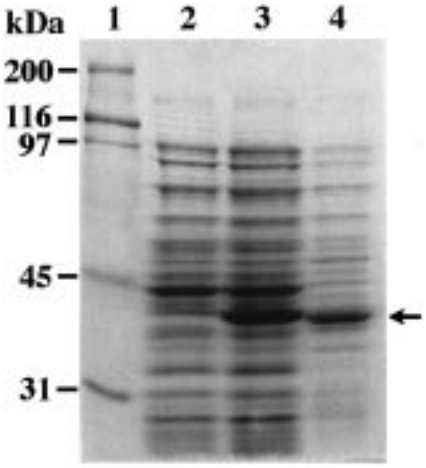

Fig. 2. SDS-PAGE analysis of soluble DAAO produced by *E. coli* BL21 (DE3) harboring pET-DAAO. Lane 1, protein size marker; lane 2, without induction; lane 3, with lactose induction; lane 4, with IPTG induction.

Results and discussion

For the expression of *T. variabilis daao* gene, *E. coli* DH5α was used as the host for the construction of expression plasmids. Plasmids pET21b-DAAO and pQE-DAAO were obtained by cloning the *daao* cDNA into plasmids pQE30 and pET21b, respectively. Sequencing analysis of plasmid-encoded *daao* gene showed that the DNA sequence of selected transformants did not alter during RT-PCR. SDS-PAGE analysis of DAAO protein produced by transformed *E. coli* BL21(DE3) is shown in Figure 2. *E. coli* BL21 (DE3) (pET-DAAO) produced a protein band with an apparent M_r of approximately 39.3 kDa.

In the presence of 1 mM IPTG, *E. coli* DH5α harboring pET-DAAO/pQE-DAAO grew slowly and resulted in a rather lower optical density than that without bearing *daao* gene. A phenomenon of elongated cells without division was observed (data not shown). The promoter used in the expression vectors usually cannot regulate tightly (Weickert *et al*. 1996) and this situation would result in the leaky expression of *T. variabilis daao* gene. The leaky expressed DAAO might consume the D-amino acids, which are important components for the synthesis of bacterial cell wall. Cells without enough cell wall compositions could not divide into two cells in the proper time and caused an elongated cell. The recombinant *E. coli* harboring pET-DAAO had less leaky expression (Table 1) than others, indicating the promoter used to express *daao* gene in pET-DAAO had a more tight regulation. This result is consistent with our previous study in expression of toxic hydantoinase gene in *E. coli* (Chien *et al*. 1998).

For alleviating the cell damage, a decreased concentration of inducer was used to optimize the induction condition. Induction with 0.4 mM IPTG for 3 h or 2 mM lactose for 7 h was found to have a better production of DAAO. Under lactose induction, the pET-DAAO-mediated expression system had a highest specific activity of 20.7 U mg^{-1} and productivity of 23.4 U ml^{-1} (Table 1). It is worth noting that DAAO protein accounting for ~15% of total soluble proteins was obtained in the crude extract of *E. coli* BL21 (DE3) (pET-DAAO) under the induction of lactose, while a less amount of enzyme was produced in the IPTG-induced cells. A variety of proteins have been expressed in recombinant *E. coli* (Donovan *et al*. 1996). The expression of target genes is initiated by the addition of IPTG and the recombinant proteins often existed within the host cell in the form of insoluble aggregates. Therefore, the phenomenon of high *T. variabilis* DAAO expression and low specific activity in IPTG-induced cells could be due to the formation of inclusion bodies.

It has been reported that a specific activity of 8.8 U mg^{-1} and a productivity of 2.3 U ml^{-1} are conducted in the expression of *R. gracilis daao* gene in *E. coli* (Molla *et al*. 1998). Using 5 mM *N*-carbamoyl-D-

alanine as the inducer, the highest productivity for *T. variabilis* DAAO is 4.2 U ml^{-1} (Hörner *et al*. 1996). In our case, a better productivity of 23.4 U ml^{-1} was achieved and the recombinant *E. coli* could grow almost normally under the lactose-induced condition. Furthermore, the DAAOs encoded by pQE-DAAO and pET-DAAO were chimeric proteins, which were fused with a six-histidine tag in C-terminus and N-terminus of native enzyme, respectively. Kinetic analysis showed that the six-histidine tag located in either N- or C-terminus did not affect the enzymatic activity of DAAO (data not shown). Therefore, the His_6-tagged DAAO would facilitate its purification from cell extracts by Ni-column chromatography.

Lactose can serve simultaneously as inducer and carbon source for the production of commercially important proteins in *E. coli* (Donovan *et al.* 1996); however, only a few reports have dealt with the use of the lactose as the inducer for foreign gene expression (Neubauer *et al.* 1992, Gombert & Kilikian 1998). Previously, we have used it successfully in the expression of toxic hydantoinase gene in *E. coli* (Chien *et al*. 1998). Due to its low cost and toxicity compared to IPTG, the use of lactose may provide an inexpensive alternative for the induction of *lac* promoter-mediated heterologous gene expression. In this study, lactose is proved to be a suitable inducer for the expression of *daao* gene under the control of the T7 promoter/polymerase system regulated by the *lac* promoter, indicating the potential for the production of *T. variabilis* DAAO by an *E. coli* expression system.

Acknowledgement

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