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High-throughput screening assay for D-amino acid oxidase

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Abstract

A membrane-based high-throughput screening (HTS) assay for active D-amino acid oxidase (DAAO) in liquid samples as well as in intact *Escherichia coli* cells has been developed and optimized. The detection limit of the assay was less than 1 ng per sample. The method proposed can be used for quantitative DAAO determination in the range of 0.13 to 3.60 ng enzyme per probe. The protocol was successfully tested to screen a library of *E. coli* clones containing mutant DAAOs active toward target substrates.

Keywords: D-Amino acid oxidase; High-throughput screening; Peroxidase reaction; Membrane; Recombinant *Escherichia coli* cells; Mutant library

D-Amino acid oxidase (DAAO,[1] EC 1.4.3.3) is an FAD-dependent enzyme that catalyzes oxidative deamination of D-amino acids yielding the corresponding α-keto acids, hydrogen peroxide and ammonia. The enzyme is of both fundamental and practical value. In microorganisms, the enzyme plays an important role in metabolism [1,2]. In mammals, its main function is the use of endogenous D-amino acids accumulated in the course of racemization. A relationship between the accumulation of D-amino acids and mammalian cell aging has been established [3,4]. Another very important role of DAAO is to support the levels of D-serine in brain tissues because this D-amino acid participates in regulation of *N*-methyl-D-aspartic acid (NMDA) receptors [5–7]. In addition, DAAO is able to effectively oxidize D-3,4-dihydroxyphenylalanine (D-DOPA), a fact that can be used in the monitoring and treatment of Parkinson's disease [8]. DAAO is of potential importance for biotechnology because it can be used to synthesize cephalosporin antibiotics [9,10], to make biosensors [11,12], to separate racemic mixtures of amino acids [13], and to produce α-keto acids [14].

One factor limiting the application of DAAO is the high catalytic activity toward only a few natural D-amino acids [15]. For example, DAAO is highly active with D-methionine, D-alanine, and D-valine and is nearly inactive toward D-aspartic and D-glutamic acids. D-Serine, D-threonine, D-lysine, and D-glycine are poor substrates. Thus, the production of mutant DAAO forms with improved activity and specificity for specified D-amino acids is a pressing problem. To obtain the enzyme forms with the desired substrate specificity, one may use various methods of directed and random mutagenesis. For the later years, mutagenesis techniques such as directed evolution and gene mosaics have become very popular. These procedures result in a variety of mutant clones and do not require detailed knowledge of the enzyme structure. Applying these methods, a large number of clones (up to a half-million) need to be screened to select the desired combination of properties [16]. Sensitivity and selectivity of the enzyme assay directly in clones define the success of high-throughput screening (HTS).

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[1] *Abbreviations used:* DAAO, D-amino acid oxidase; NMDA, *N*-methyl-D-aspartic acid; D-DOPA, D-3,4-dihydroxyphenylalanine; HTS, high-throughput screening; TvDAAO, D-amino acid oxidase from *Trigonopsis variabilis;* KPB, potassium phosphate buffer; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DAB, 3,3-diaminobenzidine; HRP, horseradish peroxidase; DMSO, dimethyl sulfoxide; IPTG, isopropyl-β-D-thiogalactopyranoside; ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline)-6-sulfonic acid.

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Currently, there are no publications available on the membrane assay for DAAO activity. Sacchi and coworkers [17] used peroxidase/*o*-dianisidine-based assay to screen the library of mutant clones. The analysis was performed on microplates. The above method is labor- and time-consuming and requires multiple handling of each clone, namely, cultivation of each clone in microplate wells, cell transfer to the assay microplates, cell lysis, and activity determination. On the contrary, membrane-based screening is simple, fast, and cost-effective.

The current work aims at developing an HTS assay for DAAO on membranes that can be applicable for the detection of microquantities of the enzyme in biological fluids and intact cell colonies.

Materials and methods

The recombinant enzyme (TvDAAO), the product of the *daao* gene from yeast *Trigonopsis variabilis,* was expressed in *Escherichia coli* cells [18].

Purification procedure

E. coli cells with recombinant DAAO were suspended in buffer A (20 mM Tris–HCl, pH 7.5) to get a 20% (w/v) suspension. Cells were disrupted with an ultrasonic disintegrator (BraundSonic, Germany). Cell debris was removed by centrifugation for 30 min at 25,400*g*. The supernatant was applied on a Mono Q HR 10/10 column (Pharmacia Biotech, Germany) equilibrated with buffer A. The enzyme was eluted at a 2.5 ml/min flow rate with a linear gradient of 0 to 1 M NaCl and buffer A. Active fractions were collected and concentrated down to 3 ml by ultrafiltration through an Amicon PM-10 membrane. The second chromatography step was gel filtration through a Sephacryl S-200 column (2.5 × 90 cm, Pharmacia Biotech) equilibrated with 0.1 M potassium phosphate buffer (KPB, pH 8.0). As judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the enzyme purity was at least 95%. The absorbance ratio (A_{280}/A_{455}) for purified enzyme was equal to 6.4.

The determination of activity and kinetic parameters of TvDAAO in solution was performed as described in Ref. [19].

TvDAAO activity assay on membranes

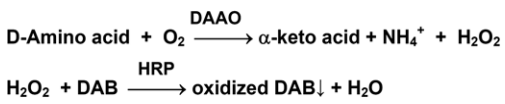
Two types of membranes, namely nitrocellulose (Bio-Rad Laboratories, UK) and polyvinylidene fluoride (Immobilon, Millipore, USA), were used. The membrane preparation included 10 to 15 min preincubation in 50% ethanol followed by washing with 0.1 M KPB (pH 8.0). Samples of various enzyme concentrations (2–3 μl) were placed on the membrane with a micro syringe. The membrane was dried in air for 2 to 3 min and then incubated at room temperature for 30 to 40 min in 0.1 M KPB (pH 7.0) containing 1% (w/v) bovine serum albumin (BSA, Sigma, USA) to block possible nonspecific binding, after which the membrane was washed in 0.1 M KPB (pH 8.0). Then the membrane was incubated in the staining solution, which contained 10 mM D-methionine (DiaM, Russia), 0.2 mg/ml 3,3-diaminobenzidine (DAB, Sigma), and 0.5 mg/ml horseradish peroxidase (HRP, Reanal, Hungary) in 50 mM KPB (pH 8.0). A stock solution of DAB (50×) was prepared in dimethyl sulfoxide (DMSO) because the solubility of DAB in water is limited.

TvDAAO activity assay in E. coli clones

Random mutagenesis of the *daao* gene was performed using *E. coli* XL1-Red competent cells (Stratagene, Germany) according to the manufacturer's protocol. Membrane replicas of Petri dishes with cell colonies of less than 1 mm in diameter were made. Then the membrane was placed on solid medium (1% agar with 2YT medium [10 g/L yeast extract, 16 g/L bactotrypton, and 5 g/L NaCl]) with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were grown for 4 to 6 h at 25 °C. Then the dish was placed in 2 to 3 ml of 0.2% (v/v) Triton X-100 in 0.1 M KPB (pH 7.0) and incubated for 5 to 10 min at 25 °C, followed by washing with 0.1 M KPB (pH 7.0). The dish was dried and covered with 1% agarose solution in 50 mM KPB (pH 8.0) containing 0.5 mg/ml HRP to obtain a layer thickness of 2 mm. HRP was added to liquid agarose when the temperature was below 45 °C. Once the agarose had solidified, the clones were activity stained as follows. The dish was covered with a solution containing D-amino acid (10 mM D-methionine, 10 mM D-tryptophan, or 30 mM D-aspartic acid), 0.2 mg/ml DAB in 50 mM KPB (pH 8.0), and incubated on mixing at 30 °C until the cells were visualized as brown–black clones.

Results and discussion

Several assay procedures for DAAO have been described in the literature, including consumption of D-amino acid monitored by HPLC, amperometric detection of oxygen or hydrogen peroxide, and ion-selective electrodes for ammonium ions [20–22]. However, these techniques cannot be used for screening cell colonies. For this purpose, a sensitive method is based on a coupled enzyme reaction with comparatively inexpensive HRP and its substrate DAB, which yields an insoluble brown–black oxidation product according to Scheme 1. DAB and its close analog, tetraaminobenzidine, provide much higher sensitivity of hydrogen peroxide detection than do the other peroxidase substrates such as 2,2′-azino-bis-(3-



Scheme 1. Peroxidase-based detection of D-amino acid oxidase.

ethylbenzthiazoline)-6-sulfonic acid (ABTS), *o*-dianisidine, guaiacol, and *o*-phenylenediamine (the last compound is carcinogenic) [23].

Our approach has the advantage of choosing any DAAO substrate of interest. In this work, we used D-methionine, a good substrate for the wild-type enzyme, for dot–blot analysis of native DAAO in liquid samples. To screen the library of *E. coli* colonies with mutant DAAOs, we tested different D-amino acids (see below).

Optimization of the assay procedure

Activity detection was performed using two types of membranes: a hydrophilic nitrocellulose membrane and a hydrophobic Immobilon membrane (see Materials and methods). The results are presented in Fig. 1. The nitrocellulose membrane leads to high sensitivity, but the drawback in this case is a high background level of the membrane itself. For the Immobilon membrane, the detection limit is low compared with that for the nitrocellulose membrane; moreover, the background is rather high. The lower sensitivity of the Immobilon membrane assay may result from hydrophobic interactions between the enzyme and membrane that can affect the enzyme structure and result in FAD dissociation from the active site. As seen in Fig. 1B, the signal intensity is almost unchanged when enzyme content is less than 60 ng. Therefore, in further experiments, we chose to use nitrocellulose membranes.

The cause of the high background for the nitrocellulose membrane might be the inability of peroxidase in solution to rapidly use hydrogen peroxide formed on the surface at the sites of DAAO binding. Hydrogen peroxide, unscavenged at the surface, diffuses into the solution and initiates the reaction in the solution volume, resulting in the precipitation of oxidized DAB over the entire membrane. To lower this nonspecific background, we decided to add HRP directly to the sample and not to the assay solution. This approach reduces the membrane background and also decreases the assay cost because the amount of enzyme added to the sample directly is significantly smaller than that added to the assay solution. The optimization of the amount of HRP added directly to the sample is presented in Fig. 2. The addition of HRP directly to the sample removes nearly all of the background coloring of the membrane. When the peroxidase amount is varied from 0.03 to 0.32 μg per sample, the intensity of the spot rises linearly, but at 0.61 μg HRP and above the intensity is almost unchanged. Accordingly, we chose 0.61 μg HRP per sample as optimal.

An additional advantage of the assay format is the avoidance of a blocking step with BSA that reduces both the assay duration and cost. The assay results of detection on a nitrocellulose membrane are shown in Fig. 3. Under the optimized conditions, there is hardly any background signal and the assay sensitivity is approximately 0.1 to 0.4 ng enzyme per sample.

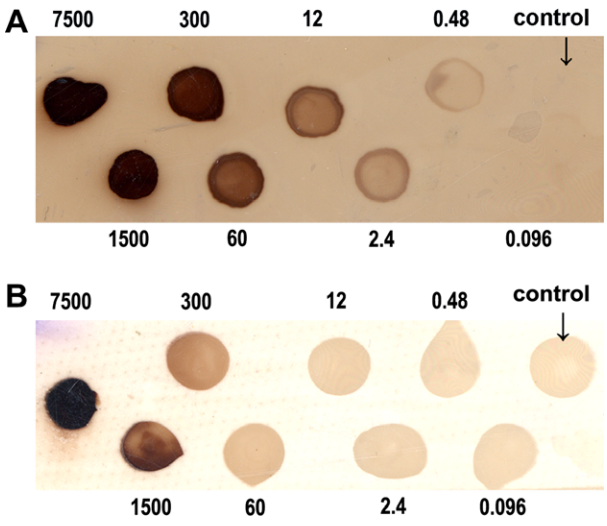


Fig. 1. Detection of DAAO (in ng) using nitrocellulose (A) and Immobilon (B) membranes. For details, see Materials and methods.

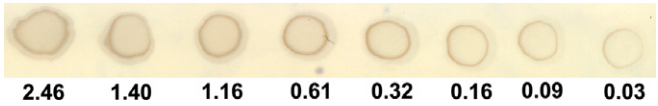


Fig. 2. Dependence of signal intensity on amount of peroxidase (in μg) added to the sample. Nitrocellulose membrane, 0.48 ng DAAO per sample.

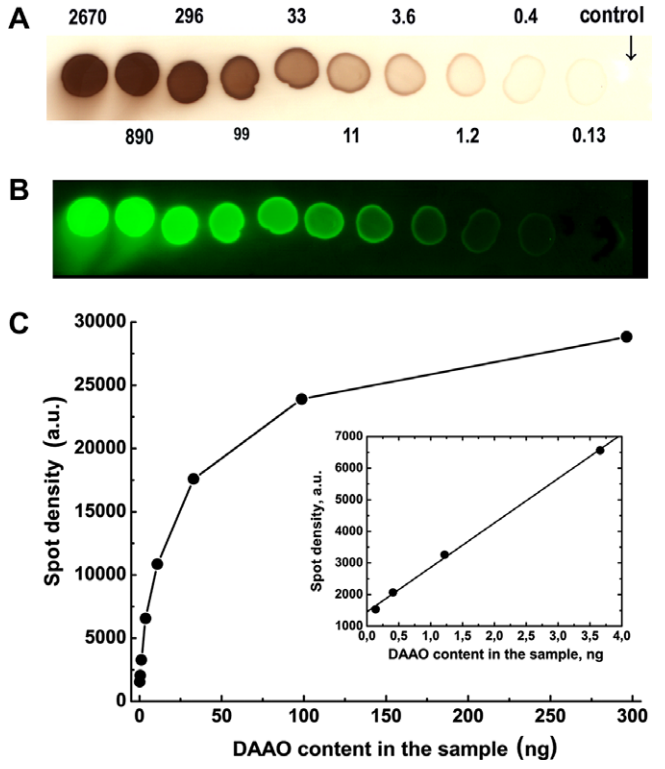


Fig. 3. DAAO detection (in ng) under optimized conditions (nitrocellulose membrane, 0.61 μg peroxidase/sample). (A) Original membrane. (B) Negative image of the membrane in grayscale. (C) Dependence of spot density on DAAO content in the sample in the range of 0.13 to 296 ng. Insert: Dependence of spot density on DAAO content in the range of 0.13 to 3.60 ng. Quantification of spot density was carried out with ScanArray 3.0 software using negative image B.

To quantify the results, the membrane was scanned (resolution 300 dpi) and the image was converted to a gray-scale format as a negative (Fig. 3B), with the spot density being determined with ScanArray 3.0 software (PerkinElmer, Germany). Fig. 3C shows the dependence of the spot density on DAAO content in the range of 0.1 to 296 ng per sample (variation of more than four orders of magnitude). At the higher enzyme content, the spot density was actually the same as for 296 ng; therefore, the data were not presented (Fig. 3A). The dependence is not linear. However, within the narrower range of 0.13 to 3.6 ng, there is a linear correlation between the spot density and DAAO content in the sample (Fig. 3C, insert). When three samples with the same content of DAAO were applied to membrane, the statistical analysis of the spot density showed a standard deviation of 18%.

Validation of the method

False positive results in the case of DAAO detection may originate from the presence of both peroxidase activity and peroxidase substrate in the sample. Peroxidase substrate can be easily removed by washing the membrane so that the background peroxidase reaction cannot proceed. False negative results may originate from catalase activity in the sample. Catalase can be easily inactivated by washing the membrane with EDTA- or EGTA-containing solution. Thus, membrane pretreatment prevents false positive and negative results.

We tested cell-free extract of *E. coli* and blood serum and did not find any DAAO activity. The intensity of background was the same as for phosphate buffer. The addition of exogenous DAAO to cell-free extracts of *E. coli* resulted in the same spot density as for an equivalent amount of pure enzyme in phosphate buffer (data not shown). Thus, the method developed here can be used to determine DAAO in various types of samples, including complex biological fluids.

Screening of mutant DAAO libraries

To evaluate the applicability of the procedure for the screening of clones with DAAO activity, one must estimate the amount of the target enzyme in a given clone.

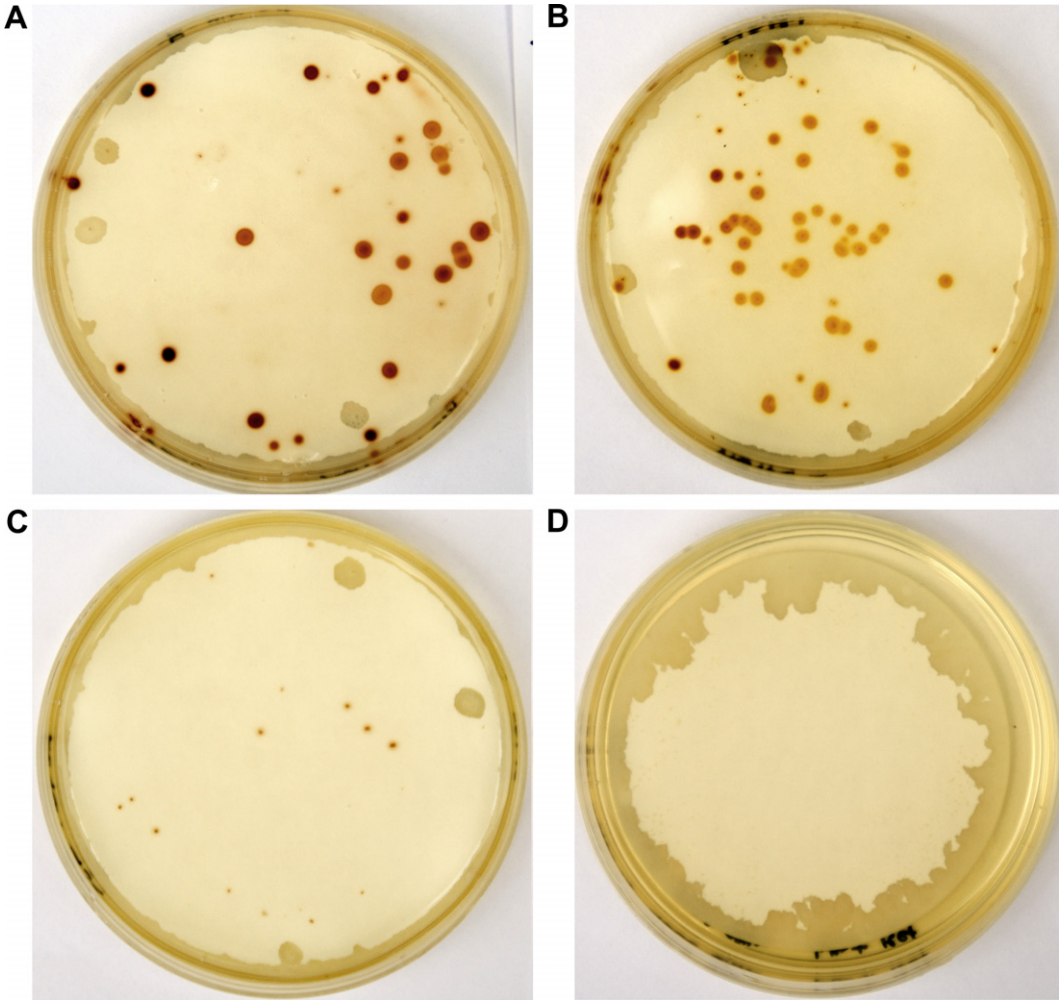


Fig. 4. Screening of mutant DAAO clones obtained by random mutagenesis using D-methionine (A), D-tryptophan (B), or D-aspartic acid (C) as substrate and a control plate with *E. coli* cells lacking the plasmid that contains the *daao* gene (D).

By assuming that each colony is a hemisphere of approximately 1 mm diameter (corresponding to 0.25 mm^3 volume and 0.25–0.30 mg wet biomass), the mass of a dry cell is approximately 15 to 20% of that of a wet cell, and the mass of a soluble protein is approximately 45% of the dry cell weight, one can calculate that each clone contains 12 to 16 μg soluble protein. Our expression system for DAAO in *E. coli* cells results in no less than 15 to 20% target protein content in the total soluble protein (i.e., one clone may contain up to 2–3 μg DAAO). Thus, keeping in mind that the sensitivity of our assay is 0.5 to 1.0 ng per sample, one can easily detect DAAO in intact *E. coli* cells at an enzyme expression level of 0.01% of cell soluble proteins.

The results obtained with the newly developed protocol for screening a mutant DAAO library using different substrates are presented in Fig. 4. DAAO detection in cell clones gives no background membrane coloring. It is likely that the absence of background is the result of agarose coverage of the membrane surface preventing hydrogen peroxide from diffusing into the solution volume. *E. coli* cells that do not contain the plasmid with the *daao* gene give no color staining (Fig. 4D). As seen in Fig. 4, different clones give distinct degrees of coloration. The data obtained are in good agreement with the substrate specificity of recombinant TvDAAO. The maximum number and intensity of colored clones was observed for D-methionine. In the case of D-tryptophan, a poor substrate of DAAO as compared with D-methionine, the intensity of clone coloring for equal incubation times in the reaction mixture is lower. For D-aspartate, which is not a substrate for the wild-type enzyme, both the number and intensity of colored clones are lower. Therefore, the developed method permits a comparison of enzyme activities in different clones and selection of the best clones for a chosen substrate for further analysis.

The main result of the current study is the elaboration of an HTS assay for microquantities of DAAO in two types of sample: biological fluids and cell clones. In the case of analysis of DAAO in fluids, the principal novelty is the addition of peroxidase directly to the sample, providing a significant decrease in the background signal and an increase in the assay sensitivity. The use of DAB as a peroxidase substrate results in the formation of an insoluble reaction product that provides a method for the successful membrane screening of up to 1500 clones from one plate. There is no manipulation of individual clones. Moreover, to screen for different DAAO substrates, one simply needs to make additional replicas. A sensitive detection of DAAO in *E. coli* cells enables the selection of clones containing DAAO mutants active with poor substrates. For example, the application of the HTS assay developed in this work allowed us to identify mutant forms of DAAO that are active toward D-aspartic acid, which is not a substrate for the wild-type enzyme (Fig. 4C).

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