Acetylcholinesterase Assay for Rapid Expression Screening in Liquid and Solid Media

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ABSTRACT

The synaptic enzyme acetylcholinesterase (AChE), which is the target of many insecticides and potential warfare agents, is implied in Alzheimer’s disease and is a good potential candidate to be used in biosensors. This promotes a strong demand for production of recombinant AChE to be used in various studies. A promising expression system is the yeast Pichia pastoris, but the expression efficiency needs to be improved. Optimization studies require a rapid and efficient screening test to detect positive yeast colonies after transformation. Using indoxylacetate as a substrate, we designed a chromogenic test that is not interfered with by the culture media background color and, thus, is suitable for microplate screening. Moreover, it was possible to adapt the test for direct on-plate detection of AChE-expressing colonies.

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INTRODUCTION

Acetylcholinesterase (AChE) is the enzyme responsible for acetylcholine hydrolysis in the synaptic cleft during nerve transmission. In addition, this enzyme plays a role in Alzheimer’s disease, and it is the target of potential warfare agents such as sarin, as well as carbamate and organophosphate insecticides. Moreover, it is possible to use AChE in sophisticated versions of biosensors to detect pesticides traces in natural samples (4,5). Such studies require the production of various recombinant wild-type AChEs, as well as mutated forms, and some authors have focused on the expression of AChE genes in different hosts (6,7,10,12–14). A promising system is the yeast Pichia pastoris, in which AChE has been successfully expressed (10,14). Nevertheless, for a rapid visual screening of positive recombinant plated colonies after transformation, the Ellman test (8) has the major drawback of giving a yellow product that is difficult to discriminate from the color of the culture media. The development of a new test is thus of major concern.

Alternative methods to detect AChE activities have been proposed (2), and among them, the chromogenic substrate indoxyl acetate has been reported to be metabolized by AChE (1–3,9, 11,15). In this study, we used this substrate to develop a rapid visual test to screen positive recombinant yeast colonies in microplates and even directly on agar plates.

MATERIAL AND METHODS

For kinetic curves, AChEs from different sources were used: AChE from human erythrocytes (HuAChE; Sigma, St. Louis, MO, USA), AChE from Electrophorus electricus (Eel AChE; Sigma), and the recombinantly expressed AChE from the nematode Nippostrongylus brasiliensis (12,13) (NbraAChE). For the expression of this AChE, a C115 P. pastoris strain (Invitrogen, Carlsbad, CA, USA) transformed with the nematode AChE-encoding gene inserted in the vector pPICaZ (12), under the methanol-inducible AOX1 promoter, was grown in shaking flasks containing YPD media (1% yeast extract, 2% peptone, 2% dextrose) supplemented with 1% glycerol. After 48 h of culture at 200-rpm agitation and 30°C, AChE expression was induced by addition of 0.5% methanol. The cells were pelleted by centrifugation for 1 min at 12000×g, and the media was used as a source of enzyme. After a 30-min incubation with the indoxyl acetate (Sigma), the absorbance was monitored at 605 nm in 25 mM phosphate buffer, pH 7.0, at room temperature after shaking of the cuvette.
A microplate test was performed by adding 4 μL stock solution of 0.1 M indoxyl acetate dissolved in dimethyl sulfoxide (DMSO) to wells containing 200 μL raw culture media containing the nematode AChE, previously produced in shaking flasks. For comparison, the Ellman test was performed on the same enzyme dilutions with $3 \times 10^{-4}$ M 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Riedel-de-Haen) and 1 mM acetylthiocholine (ACh). The reaction time was 30 min, and controls were performed either using a culture of nontransformed C115 *P. pastoris* strain or using the AChE inhibitor paraoxon ethyl (Riedel-de-Haen). One unit of AChE is defined as the amount of enzyme necessary to give 1 A$_{412}$ nm/min in 1 mL 25 mM phosphate buffer, pH 7.0, containing 1 mM ACh, at 25°C.

For the on-plate detection test, petri dishes filled with LB medium supplemented with 15 g/L agar were first prepared with 100 μL methanol. The plate was then spread with 100 μL 0.1 M indoxyl acetate solution or with 200 μL 25 mM phosphate buffer containing 1 mM ACh and $3 \times 10^{-4}$ M DTNB. Then, the *P. pastoris* strain expressing the nematode gene and the wild-type C115 strain were plated, and the petri dishes were placed for three days at 30°C.

**RESULTS AND DISCUSSION**

To detect AChE activity, the Ellman test is based on a two-step reaction: (i) the ACh is hydrolyzed by AChE, giving the product thiocholine and (ii) the yellow compound nitrobenzozate is formed due to the reaction between DTNB and thiocholine (Figure 1). Conversely, indolyl is produced in one step, following the ester bond cleavage of indoxyl acetate and gives a green-blue color. This color is more visible than yellow for the naked eye and can also be detected spectrophotometrically at 605 nm. First, we investigated the optimum indoxyl acetate concentration for the test: the hydrolysis curves of Figure 2 show that the maximal hydrolysis velocity by AChE was achieved at 2 mM indoxyl acetate for the three AChEs tested (Figure 2). These data suggested that 2 mM represents the optimal concentration for the test. In addition, it appeared that the intensity of the color was proportional to the amount of enzyme (Figure 3), suggesting that an approximation of the enzyme amount is possible.

As this test is designed for direct detection of AChE activity in the culture media, we added directly 1 mM indoxyl acetate to culture samples containing a *P. pastoris* strain expressing the *N. brasiliensis* AChE after induction by methanol. For comparison, the Ellman test was performed under the same conditions with 1 mM ACh, which seems to be the maximal velocity concentration for several AChEs (16). Figure 4 shows the AChE activity revealed by indoxyl acetate hydrolysis and ACh hydrolysis. While it is very difficult to detect a difference between the control (wild-type cells) and the Ellman test (with transformed cells), the value of the indoxylacetate test is obvious. The dark blue-green color is better detected by the naked eye, compared to the yellow color, which is interfered with by the background color of the culture media. The metabolism of the indoxyl acetate by the AChE is confirmed by the lack of color in the controls (nontransformed *P. pastoris* strain and transformed strain plus paraoxon ethyl). This result also confirms the usual observation that yeast do not secrete any endogenous general esterases that could interfere with the

![Figure 2. Hydrolysis of indoxyl acetate by *E. coli*, human erythrocytes, and *N. brasiliensis* AChE as a function of substrate concentration. The absorbance was measured after a 30-min incubation at 605 nm in 25 mM phosphate buffer, pH 7.0, at 25°C for different indoxyl acetate concentrations.](image)

![Figure 3. Plots of absorbance at 605 nM versus *N. brasiliensis* AChE concentration at 2 mM indoxyl acetate in 25 mM phosphate buffer, pH 7.0, at 25°C.](image)

![Figure 4. Visual aspect of culture media containing *P. pastoris* cells secreting *N. brasiliensis* AChE with the Ellman test and the indoxyl acetate test. Lane 1, 2 mM indoxyl acetate plus *P. pastoris* strain expressing AChE; lane 2, 1 mM ACh plus $3 \times 10^{-4}$ M DTNB plus *P. pastoris* strain expressing AChE; lane 3, 2 mM indoxyl acetate plus wild-type *P. pastoris*; lane 4, 1 mM ACh plus $3 \times 10^{-4}$ M DTNB plus wild-type *P. pastoris*; lane 5, 2 mM indoxyl acetate plus *P. pastoris* strain expressing AChE plus 2 mM paraoxon ethyl; lane 6: 1 mM ACh plus $3 \times 10^{-4}$ M DTNB plus *P. pastoris* strain expressing AChE plus 2 mM paraoxon ethyl. (A) 3 U AChE; (B) 7 U AChE; (C) 14 U AChE; (D) 30 U AChE.](image)
We extended the principle further to a simple on-plate detection of the P. pastoris colonies expressing AChE. Figure 5 shows colonies transformed with the nematode AChE gene and the wild-type C115 colonies. The brown color due to the indoxyl acetate hydrolysis by the expressed AChE enabled the distinction from the white color of the wild-type strain. The growth difference between the transformed and wild-type strain could be due to the AChE encoding gene insertion in the genome or its expression. Such a discrimination was impossible with the Ellman test (Figure 5). Consequently, this test allows a rapid discrimination of the colonies directly on the agar plate containing the simple on-plate detection of the insecticidal nematode AChE expression. Such a discrimination was impossible with the Ellman test (Figure 5).

**REFERENCES**


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