15. **Pollard, H., J.S. Remy, G. Loussouarn, S. Demolombe, J.P. Behr, and D. Escande.** 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J. Biol. Chem. *273*:7507-7511.
16. **Remy, J.-S., B. Abdallah, M.A. Zanta, O. Boussif, J.-P. Behr, and B. Demeneix.** 1998. Gene transfer with lipospermines and polyethylenimines. Adv. Drug Del. Rev. *30*:85-95.
17. **Ryser, H.J.P**. 1967. A membrane effect of basic polymers dependent on molecular size. Nature *215*:934-936.
18. **Tang, M.X. and F.C. Szoka.** 1997. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther. *8*:823-832.
19. **Vassault, A.** 1983. Lactate dehydrogenase: UV-method with pyruvate and NADH, 118-126. *In* H.U. Bergmeyer, J. Bergmeyer, and M. Grassi (Eds.), Methods of Enzymatic Analysis, Vol. III, Verlag Chemie, Academic Press, New York.
20. **Wagner, E.** 1998. Effects of membrane-active agents in gene delivery. J. Controlled Rel. *53*:155-158.

This work was supported by the P.E. Kempkes Foundation, Marburg, and by the Dr. Hilmer Foundation, Essen. *Address correspondence to Dr. H.P. Elsässer, Department of Cell Biology, Robert-Koch Str. 5, 35033 Marburg, Germany. e-mail: elsaesse@mailer.uni-marburg.de*

Received 11 May 2000; accepted 28 August 2000.

Thorsten Bieber and Hans-Peter Elsässer
University of Marburg
Marburg, Germany

Acetylcholinesterase Assay for Rapid Expression Screening in Liquid and Solid Media

BioTechniques 30:81-86 (January 2001)

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.*

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 pPICαZ (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 12 000× *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.

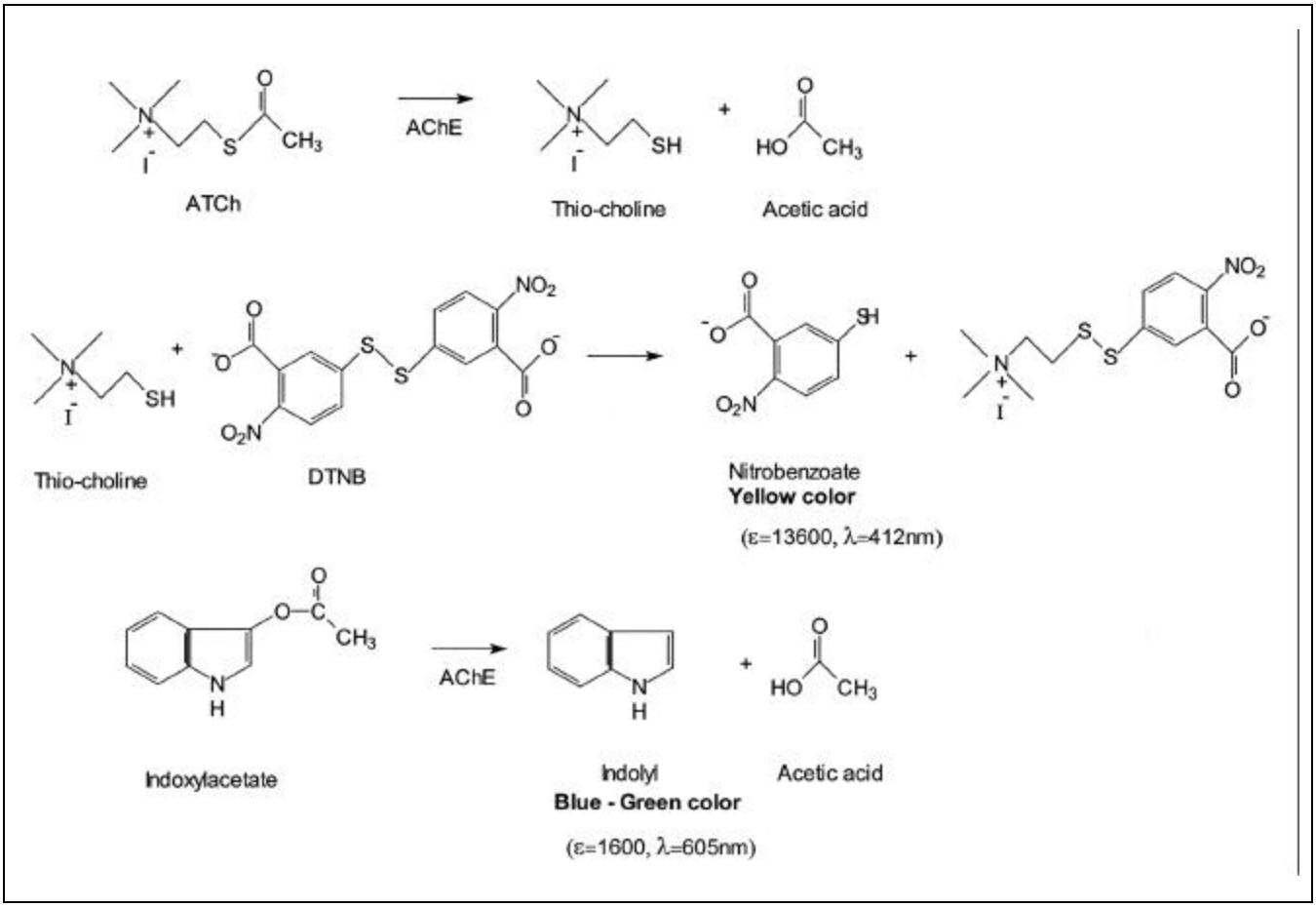

Figure 1. Substrates metabolism schemes.

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 3×10^{-4} M 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma) and 1 mM acetylthiocholine (ASCh) (Sigma). 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 ASCh, at 25°C.

For the on-plate detection test, petri dishes filled with LB medium supplemented with 15 g/L agar were first supplemented 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 ASCh and 3×10^{-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 ASCh is hydrolyzed by AChE, giving the product thiocholine and *(ii)* the yellow compound nitrobenzoate 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 ASCh, which seems to be the maximal velocity concentration for several AChEs (16). Figure 4 shows the AChE activity revealed by indoxyl acetate hydrolysis and ASCh 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

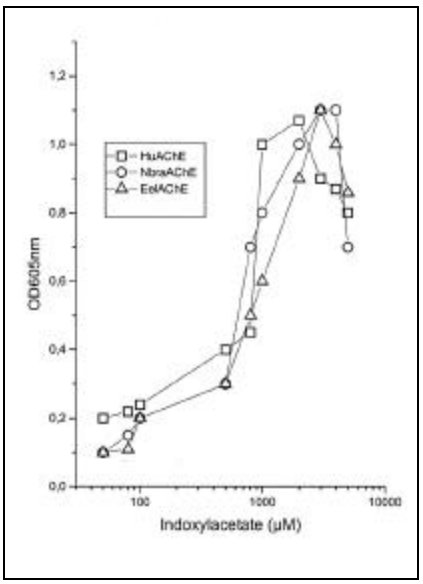

Figure 2. Hydrolysis of indoxyl acetate by *E. electricus*, human erythrocytes, and *N. brasiliensis* AChE as a function of substrate concentration. The absorbance was measured after a 30-min incubation at 605 nm in 25 mM phosphate buffer, pH 7.0, at 25°C for different indoxyl acetate concentrations.

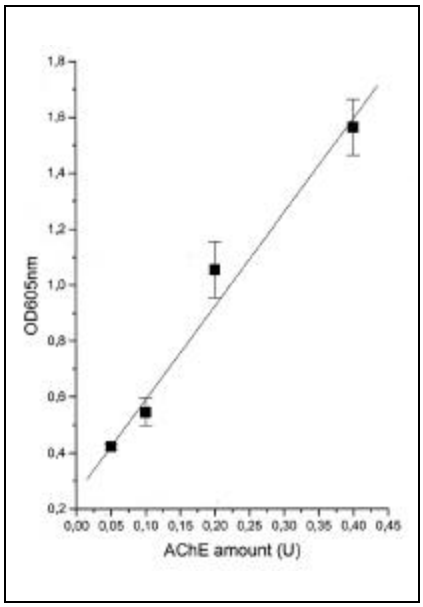

Figure 3. Plots of absorbance at 605 nM versus *N. brasiliensis* AChE concentration at 2 mM indoxyl acetate in 25 mM phosphate buffer, pH 7.0, at 25°C.

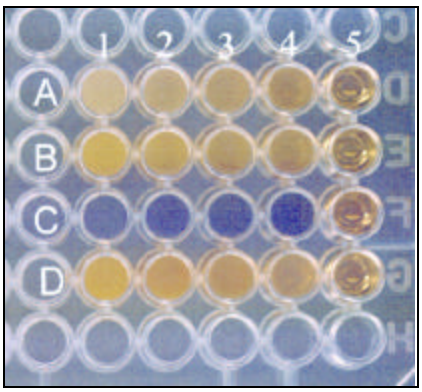

Figure 4. Visual aspect of culture media containing *P. pastoris* cells secreting *N. brasiliensis* AChE with the Ellman test and the indoxyl acetate test. Lane 1, 2 mM indoxyl acetate plus *P. pastoris* strain expressing AChE; lane 2, 1 mM ASCh plus 3×10^{-4} M DTNB plus *P. pastoris* strain expressing AChE; lane 3, 2 mM indoxyl acetate plus wild-type *P. pastoris*; lane 4, 1 mM ASCh plus 3×10^{-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 ASCh plus 3×10^{-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.

test. It is thus possible to detect rapidly the AChE-expressing colonies using indoxyl acetate as substrate. If the cell density is measured, the method can enable the identification of the highest secretion clones because the intensity of the color is proportional to the amount of enzyme (Figure 3).

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 and then enables a quick and efficient selection of the best clones to be grown in liquid media. This two-step screening strategy should contribute to the improvement of AChE expression in yeast.

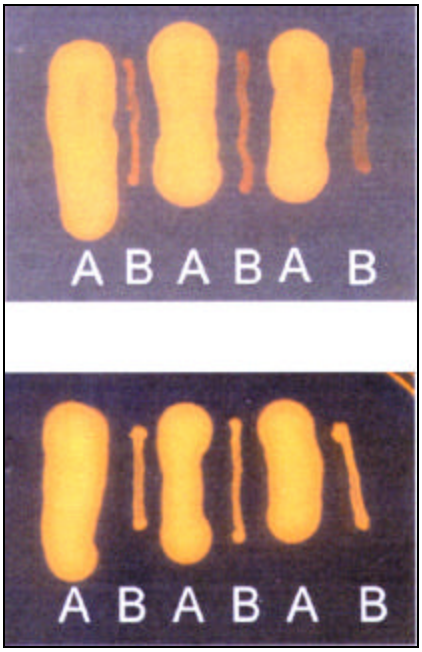

Figure 5. Visual aspect of *P. pastoris* colonies on an agar plate containing (top) indoxyl acetate [(A) no AChE secretion and (B) AChE secretion] and (bottom) ASCh and DTNB. Agar-filled petri dishes were supplemented with 100 µL methanol (top and bottom) and then with 100 µL indoxyl acetate (0.1 M) (top) or 200 µL 25 mM phosphate buffer, pH 7.0, containing 1 mM ASCh and 3×10^{-4} M DTNB.

REFERENCES

1. **Abad, J.M., F. Parientez, L. Hernandez, H.D. Abruna, and E. Lorenzo.** 1998. Determination of organophosphorus and carbamate pesticides using a piezoelectric biosensor. Anal. Chem. *70*:2848-2855.
2. **Andersen, S., K. Pecorella, P. Masson, J.P. Toutant, and J. Grassi.** 1992. Colorimetric determination of cholinesterase activity. New methods leading to the formation of soluble and insoluble end-products. Abstracts of the 36th Oholo conference, Eilat, Israel.
3. **Arpagaus, M., P. Richier, J.B. Berge, and J.P. Toutant.** 1992. Acetylcholinesterases of the nematode *Steinernema carpocapsae*. Characterization of two types of amphiphilic forms differing in their mode of membrane association. Eur. J. Biochem. *207*:1101-1108.
4. **Bachmann, T.T., B. Léca, F. Villatte, J.L. Marty, D. Fournier, and R.D. Schmid.** 2000. Improved multianalyte detection of organophosphates and carbamates with disposable multielectrode biosensors using recombinant mutants of *Drosophila* acetylcholinesterase and artificial neural networks. Biosens. Bioelectron. *15*:193-201.
5. **Bachmann, T.T. and R.D. Schmid.** 1999. A disposable multielectrode biosensor for rapid simultaneous detection of the insecticides paraoxon and carbofuran at high resolution. Anal. Chem. Acta *401*:95-103.
6. **Chaabihi, H., D. Fournier, Y. Fedon, J.P. Bossy, M. Ravallec, G. Devauchelle, and M. Cerutti.** 1994. Biochemical characterization of *Drosophila melanogaster* acetylcholinesterase expressed by recombinant baculoviruses. Biochem. Biophys. Res. Commun. *203*:734-742.
7. **Choumet, V., X. Cousin, and C. Bon.** 1999. Production of an immunoenzymatic tracer combining a scFv and the acetylcholinesterase of *Bungarus fasciatus* by genetic recombination. FEBS Lett. *455*:18-22.
8. **Ellman, G.L., K.D. Courtney, V. Andres, and R.M. Featherstone.** 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. *7*:88-95.
9. **Guibault, G.G. and D.H. Kramer.** 1965. Resorufin butyrate and indoxyl acetate as fluoregenic substrates for cholinesterase. Anal. Chem. *37*:120-123.
10. **Heim, J., C. Shmidt-Dannert, H. Atomi, and R. Schmid.** 1997. Functional expression of a mammalian acetylcholinesterase in *Pichia pastoris*: comparison to acetylcholinesterase, expressed and reconstituted from *Escherichia coli*. Biochim. Biophys. Acta *1396*:306-319.
11. **Holt, S.J. and R.F.J. Withers.** 1958. Studies in enzyme cytochemistry. An appraisal of indigogenic reactions for esterase localization. Proc. R. Soc. B *148*:520-532.
12. **Hussein, A.S., M.R. Chacon, A.M. Smith, R. Tosado-Acevedo, M.E. and M.E. Selkirk.** 1999. Cloning, expression and properties of a nonneuronal secreted acetylcholinesterase from the parasitic nematode *Nippostrongylus brasiliensis*. J. Biol. Chem. *274*:9312-9319.
13. **Hussein, A.S., A.M. Smith, M.R. Chacon, and M.E. Selkirk.** 2000. Determinants of substrate specificity of a second non-neuronal secreted acetylcholinesterase from the parasitic nematode *Nippostrongylus brasiliensis*. Eur. J. Biochem. *267*:1-8.
14. **Morel, N. and J. Massoulié.** 1997. Expression and processing of vertebrate acetylcholinesterase in the yeast *Pichia pastoris*. Biochem. J. *328*:121-129.
15. **Strum, J.M. and E.C. Hall-Craggs.** 1982. A method demonstrating motor endplates for light and electron microscopy. J. Neurosci. Methods *6*:305-309.
16. **Villatte, F., V. Marcel, S. Estrada-Mondaca, and D. Fournier.** 1998. Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides. Biosens. Bioelectron. *13*:157-164.

This work was supported by a European Union (VI framework) post-doctoral grant to F.V. Address correspondence to Dr. Rolf D. Schmid, Institute for Technical Biochemistry, Allmandring 31, Stuttgart University, 70569 Stuttgart, Germany. e-mail: rolf.d.schmid@rus.uni-stuttgart.de

Received 15 May 2000; accepted 5 September 2000.

Francois Villatte, Till T. Bachman, Ayman S. Hussein[1], and Rolf D. Schmid
Stuttgart University
Stuttgart, Germany
[1]Imperial College of Science, Technology and Medicine
London, UK