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Production of an immunoenzymatic tracer combining a scFv and the acetylcholinesterase of *Bungarus fasciatus* by genetic recombination

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Abstract We constructed a plasmid containing a chimeric gene composed of the gene encoding acetylcholinesterase (AChE) from *Bungarus fasciatus* venom and a gene encoding a single chain antibody fragment (scFv) directed against one of the two subunits of a presynaptic neurotoxin from rattlesnake. Large quantities of the fusion protein were produced in the culture medium of transfected COS cells. Fusion to AChE did not affect the ability of the scFv to recognise its antigen. Similarly, the AChE activity was not impaired in the fusion. The fusion protein was purified from the culture medium in a single step by affinity chromatography. The immunoconjugate obtained consisted of a soluble monomeric form of AChE fused to scFv. It was monovalent and had a molecular weight of 94 kDa. The properties of this scFv-AChE fusion show that the simple, reproducible preparation of various recombinant monovalent immunoenzymatic tracers with low molecular weight is possible. In addition, in the construct presented, the scFv domain can be easily changed to another one taking advantage of the *Sfi*I-*Not*I restriction sites surrounding this domain.

Key words: Acetylcholinesterase; Single chain antibody fragment; Enzymatic tracer; Recombinant fusion protein; Colorimetry; Enzyme-linked immunosorbent assay; Immunoconjugate

1. Introduction

Immunoenzymatic techniques using solid supports, particularly enzyme-linked immunosorbent assays (ELISA), are widely used for the determination in vitro of many biological substances [1–3]. In principle, numerous enzymes can be used for producing the immunoenzymatic tracers. Actually, since the enzyme assay must be simple, sensitive and accurate, the choice of enzyme is limited. In addition, the enzyme must be stable in the media containing the samples and must not interact in a non-specific manner with the solid phase or the proteins present in the sample. The enzyme used must also be easy to obtain in large quantities and at low cost, to make the process economically viable. In practice, four enzymes, horseradish peroxidase, alkaline phosphatase, β-galactosidase and acetylcholinesterase (AChE), are commonly used for ELISA [4,5]. AChE has many advantages over the other enzymes. Its catalytic constant is higher than those of the other enzymes: 14 000 s^{-1} in comparison to 2000 s^{-1} for alkaline phosphatase, 250–500 s^{-1} for β-galactosidase and 1000 s^{-1} for horseradish peroxidase [6–8]. Further, assay for AChE activity is simple, the signal increases linearly with time and there is no need to stop the reaction, as is necessary for peroxidase or alkaline phosphatase [9]. This makes it possible to adapt tests to the concentration of the substance in the sample, simply by changing the times at which readings are taken. High concentrations can be determined rapidly and lower concentrations after a longer incubation time. AChE has already been used successfully in ELISA to determine the concentrations of various substances in homogeneous and heterogeneous phases [9–13].

The preparation of immunoenzymatic tracers requires coupling an antigen or an immunoglobulin to the enzyme used for the assay. In almost all cases, this coupling is achieved chemically and specific protocols, sometimes difficult to refine and optimise, are required for each tracer. The coupling process must be repeated for each batch, and therefore the different batches will have different characteristics and have to be tested. Highly purified reagents are also required, to ensure that there is no non-characterised coupling which could lead to irrelevant results. The construction of fusion genes allowing the production of recombinant proteins in cell-based systems is an approach which avoids many of the difficulties associated with the chemical preparation of immunoreactive tracers. Immunoenzymatic tracers have already been prepared using these techniques: genes for antibodies of various sizes (Fab or scFv) have been fused to the gene encoding alkaline phosphatase to produce bifunctional conjugates linking an scFv or Fab with both catalytic subunits of alkaline phosphatase [14].

AChE from the venom of the snake *Bungarus fasciatus* has recently been characterised [15–17]. It is a soluble monomeric protein, in contrast to the other known AChE which are homo- or hetero-oligomers, soluble or attached to membranes via glycolipids or structural proteins (dimers and tetramers) [18,19]. The catalytic constant of AChE of *B. fasciatus* is lower than that of tetrameric AChE from *Electrophorus electricus* (7000 s^{-1} for *B. fasciatus* compared to 14 000 s^{-1} for *E. electricus* AChE) but higher than those of horseradish peroxidase and alkaline phosphatase. The *B. fasciatus* AChE gene is also expressed more efficiently by COS cells than AChE genes of other species [15,19].

In this work, we describe the use of genetic engineering techniques to produce a fusion protein containing a scFv directed against the CA subunit of crotoxin, the major toxic protein of the venom of the South-American rattlesnake, *Crotalus durissus terrificus* [20] and the monomeric AChE of *B. fasciatus*. The fusion between the two proteins does not affect the catalytic rate of the enzyme or the ability of the scFv to recognise its antigen. We therefore demonstrate the potential value of this system for the preparation of monovalent immunoenzymatic tracers for ELISA.

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2. Materials and methods

2.1. Materials

Acetylthiocholine and 5,5′-dithio-bis(2-nitrobenzoic acid) were purchased from Sigma (St. Louis, MO, USA). The enzymes used for plasmid constructions were obtained from Boehringer-Mannheim (Germany) and Promega Biotech (Madison, WI, USA). Monoclonal anti-c-myc 9E10 antibody was obtained from Pharmingen (San Diego, CA, USA). Crotoxin and the CA subunit of crotoxin were purified from the venom of *C. durissus terrificus* as previously described [21,22]. Other reagents and solvents were purchased from Merck (Darmstadt, Germany), Sigma (St. Louis, MO, USA) or Prolabo (Paris, France). Microtitration plates were supplied by Nunc (Roskilde, Denmark). VX (*O*-ethyl-*S*-[2-(diisopropylamino)ethyl] methylphosphothioate) was a gift of Daniel Froment (Centre d'Etudes du Bouchet, Vert-le-Petit, France).

2.2. Production of the chimeric gene encoding AChE and scFv

A cDNA encoding the AChE from the venom of *B. fasciatus* was isolated from a cDNA library in pT3T7 (Pharmacia), constructed using the *Eco*RI and *Not*I sites [15]. A fragment encoding the catalytic domain (nucleotides 1–1698) was ligated between the *Eco*RI and *Not*I sites of pCDNA3 (Invitrogen) to give pC-AChE, which was used as a control [15]. The chimeric gene was produced by PCR, using a primer with a sequence identical to that of nucleotides 1438–1459 and an antisense primer complementary to the sequence of the AChE gene of *B. fasciatus* (1884–1901), containing the coding sequence for the c-myc polypeptide and the *Sfi*I restriction site (Fig. 1A). The amplified fragment was inserted into pGEM-T (Promega) and sequenced. It was then ligated between the *Bgl*II and *Not*I sites of pT3T7, downstream of the cDNA encoding AChE. The *Bam*HI-*Not*I fragment was removed from this plasmid and ligated into pC-AChE, replacing the 3′ fragment of the truncated cDNA, to produce pC-AChE-c-myc. The gene encoding scFv I12, the antibody fragment specific for the CA subunit of crotoxin [20], was ligated between the *Sfi*I and *Not*I sites of pC-AChE-c-myc to produce pC-AChE-scFv, a plasmid which thus contains the chimeric gene (Fig. 1B).

2.3. Transfection of COS cells

COS cells were transfected with 5 μg of pC-AChE-scFv, using the DEAE-dextran method, as described by Bon and Massoulié [23]. The cells were then cultured at 37°C for 5 days and collected by centrifugation (4000 rpm for 10 min). The supernatant was removed and saved. The cells were suspended in 500 μl phosphate-buffered saline (PBS) containing protease inhibitors (Complete, Boehringer) and subjected to sonication for 90 s. Intact cells were pelleted by centrifugation, and the supernatant (cell extract) was removed and stored at 4°C.

2.4. Purification of the fusion protein

The AChE-scFv fusion protein was purified by affinity chromatography, as described by Massoulié and Bon [23]. An affinity gel for AChE was prepared by coupling the ligand, *m*-carboxyphenyldimethylethyl, to EAH Sepharose 4B [24]. The culture medium of COS cells (3 ml) was dialysed against 0.1 M potassium phosphate, pH 7.4 (buffer I) and added to a column containing 1 ml of the affinity gel, equilibrated with the same buffer. The column was washed with 2 ml of buffer I, then with 2 ml of buffer I containing 0.25 M NaCl. It was then eluted with 2 ml of buffer I containing 0.25 M NaCl and 20 mM decamethonium. The fractions with AChE activity were pooled and dialysed against 0.05 M potassium phosphate buffer, pH 7.4, for 72 h at 4°C.

2.5. Measurement of AChE activity

AChE activity was determined by colorimetry with Ellman's reagent [25]. The specific activity was determined as a function of residual activity after incubation of enzyme with several concentrations of VX as described previously [15].

2.6. Detection of the fusion protein by ELISA

Microtitration plates were coated by incubation with the CA subunit of crotoxin (100 μl per well of a 1 μg/ml solution in PBS) for 2 h at 37°C. They were then saturated using 100 μl per well of 0.1% Tween 20, 3% bovine serum albumin (BSA) (buffer II). Supernatants from COS cultures and cell extracts were serially diluted in buffer II. 100 μl aliquots of each dilution was added to each well and the plates were incubated for 1 h at 37°C. Two detection methods were used. AChE activity was detected by adding 100 μl Ellman's 2× reagent per well and incubating for various periods of time. Optical density at 415 nm was then measured. The fusion protein was detected by adding 100 μl of a 1 in 500 dilution in buffer II of monoclonal antibody 9E10, specific for the c-myc polypeptide, to each well and incubating for 1 h at 37°C. The plate was washed with PBS containing 20% Tween 20 (PBS-Tween) and incubated with a peroxidase-conjugated anti-mouse IgG antibody. The plate was washed again, incubated with the substrate, *o*-phenylenediamine (OPD), and optical density measurements were performed at 490 nm.

2.7. Affinity of the fusion protein

The fusion protein, AChE-scFv, was incubated at an appropriate dilution (as assessed from the direct ELISA experiments) with various concentrations of the CA subunit, overnight at 4°C. Aliquots (100 μl) were transferred to a microtitration plate coated with CA and incubated for 30 min at 37°C. The plate was washed with PBS-Tween and residual AChE activity was determined as described above, after incubation of the plate for 4 h at 37°C.

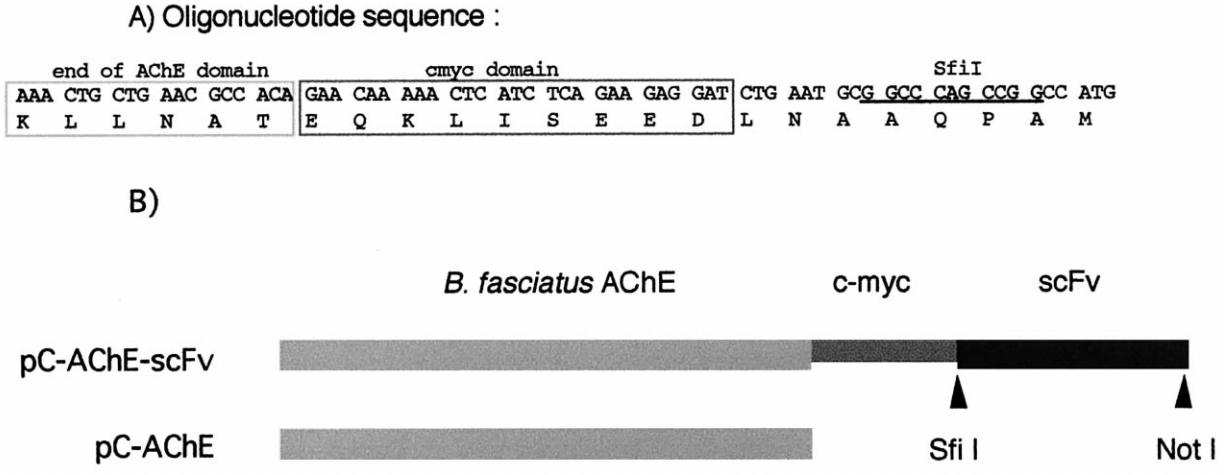

Fig. 1. Construction of the AChE-scFv conjugate. A: Oligonucleotide used to produce the chimeric gene. B: Construct transfected in COS cells. pC-AChE-scFv encodes a fusion protein associating *B. fasciatus* AChE with a scFv anti-CA. These domains are separated by a c-myc peptide. The pC-AChE construct encodes AChE restricted to the catalytic domain and was used as a control.

2.8. Analysis of the fusion protein by polyacrylamide gel electrophoresis (PAGE)

The solution containing the affinity-purified protein was concentrated by centrifugation on a Centricon slide (Amicon, Grasse, France) and subjected to SDS-PAGE in a 12.5% acrylamide gel with a Phastsystem apparatus (Pharmacia, Uppsala, Sweden). The gel was stained using the colloidal Coomassie kit (Novex, San Diego, CA, USA).

3. Results

3.1. Construct encoding the AChE-scFv fusion protein and expression in COS cells

A scheme of the synthetic gene construct encoding the AChE-scFv fusion protein is shown in Fig. 1B. *B. fasciatus* AChE undergoes a post-translational cleavage removing the last eight residues before secretion by COS cells. For this reason, the AChE domain of the fusion protein is restricted to the catalytic domain and ends seven amino acids upstream of the cleavage site. The c-myc domain is recognised by the 9E10 antibody, facilitating the detection of the fusion protein. The scFv domain encodes an antibody that recognises the CA subunit of crotoxin [20]. The scFv domain is inserted between *Sfi*I-*Not*I restriction sites. This allows an easy change of this domain and the use of this construct with virtually any cloned scFv.

The production of the AChE-scFv fusion protein by transfected COS cells was checked by measuring AChE activity in the culture medium and in cell extracts obtained by sonication of the cells (Table 1). We used pC-AChE as a positive control and pCDNA3 with no insert as a negative control for the transfection of COS cells. Low levels of AChE activity were detected in the supernatants of cells transfected with the vector lacking an insert, presumably due to the secretion of the native AChE of COS cells or to the AChE activity of the serum used for the cell growth and remaining in the transfection medium. Higher levels of AChE activity were detected in cells transfected with pC-AChE-scFv or pC-AChE.

3.2. Functional properties of the AChE-scFv fusion protein

The AChE-scFv fusion protein was detected in cell extracts by ELISA. The microtitration plate was coated with the CA subunit and anti-c-myc antibody 9E10 was used for detection (Fig. 2A). The negative control was supernatant of cells transfected with pC-AChE and disrupted by sonication. A protein containing scFv and the c-myc peptide was clearly present in the culture medium of COS cells transfected with pC-AChE-scFv. No such protein was detected on the supernatant of cells transfected with pC-AChE. Similar results were obtained using culture supernatants of transfected COS cells (results not shown).

Table 1
AChE activity measurement in COS cell extracts and culture supernatants

Vector	Enzymatic activity (U/ml)	Deduced concentration (μg/ml)
Cell extracts		
pCDNA3	8.5 ± 1.14	0.112 ± 0.015
pCB1	30.3 ± 4.36	0.401 ± 0.057
pC-AChE-scFv	74.56 ± 7.14	0.985 ± 0.09
Culture supernatants		
pCDNA3	0.03 ± 0.005	0.44×10^{-3}
pcB1	52.2 ± 0.07	0.691 ± 0.0009
pC-AChE-scFv	102.1 ± 0.014	1.36 ± 0.00018

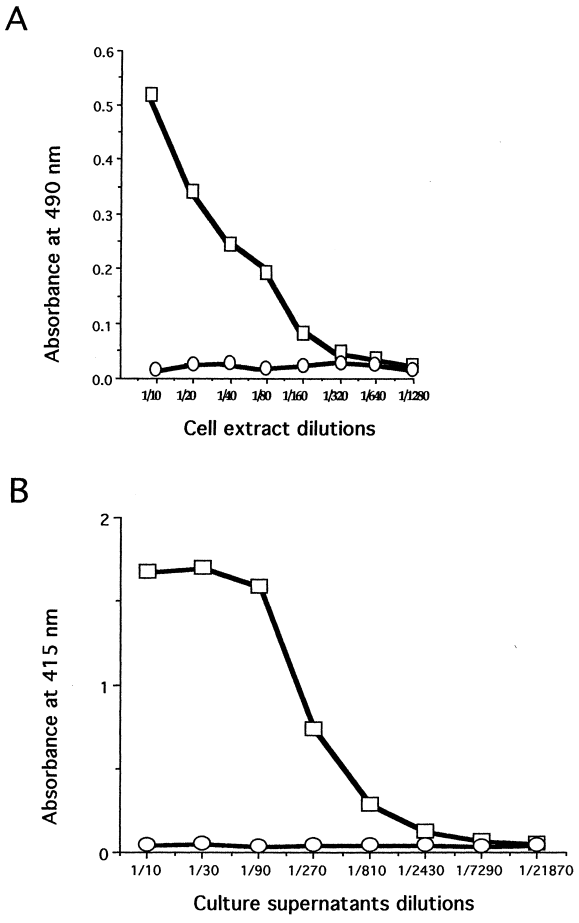

Fig. 2. Function of the immunoenzymatic tracer, AChE-scFv. Culture medium was incubated in a microtitration plate coated with the CA subunit as described in Section 2. The conjugate was revealed by 9E10 anti-c-myc antibody (A) or directly by AChE activity (B). (○) pC-AchE; (□) pC-AChE-scFv. To allow comparison, the amount of conjugate was normalised according to AChE activity. Note that the scale of each graph is different.

The fusion protein was also detected using an alternative ELISA test in which the microtitration plate was coated with the CA subunit and the protein was detected by direct measurement of AChE activity. The fusion protein was specifically detected in COS cells transfected with pC-AChE-scFv, even at a dilution of the conjugate as high as 1 in 2000 (Fig. 2B). This detection method was much more sensitive than detection with the anti-c-myc antibody which can only detect the conjugate in dilutions of 1 in 180 or lower (Fig. 2A). Thus, the AChE activity measured was associated with scFv, which recognised the CA subunit bound to the microtitration plate. AChE activity as determined by these ELISA assays was inhibited in a dose-dependent manner by the CA subunit, with 50% inhibition at a CA subunit concentration of 5×10^{-9} M (Fig. 3). The IC_{50} of CA in the AChE-scFv complex was identical to the affinity of the parental antibody, A-56.36, for CA and that of free scFv [20,26]. This shows that the affinity of scFv I12 for CA was not affected by fusion to AChE. The catalytic properties of *B. fasciatus* AChE do not seem to be modified by the association of this enzyme with a scFv since the turnover number of the immunoenzymatic trac-

er is identical to that of recombinant AChE, 6700 ± 30 s^{-1} ($n = 3$) compared to 6600 ± 80 s^{-1}, respectively.

3.3. Purification of the AChE-scFv fusion protein and PAGE analysis

We purified the AChE-scFv fusion protein from the cell culture supernatants of COS cells transfected with pC-AChE-scFv in a single step, by AChE affinity chromatography. The purification yield was 40%. The purified fraction gave a single protein band in SDS-PAGE, showing that the fusion protein can be effectively and simply purified from cell culture medium (Fig. 4). The estimated molecular weight of this protein was slightly higher than 94 kDa, consistent with the molecular weight of the fusion protein (97 kDa) calculated from the molecular weight of its components, scFv I12 (25 kDa) and AChE (72 kDa).

4. Discussion

We produced a fusion protein containing the AChE of *B. fasciatus* linked by a c-myc peptide to an scFv which recognises the CA subunit of crotoxin. This fusion protein was efficiently secreted in the culture medium by transfected COS cells. The fusion protein was active: via scFv it binds specifically to the CA subunit coated onto microtitration plates and is detected by measuring AChE activity. Fusion of scFv to AChE did not affect its ability to recognise the antigen, because the affinity of the fusion protein (5×10^{-9} M) is similar to that previously determined for scFv and the parental immunoglobulin [26,20]. Ducancel et al. [14] obtained a fusion protein containing an scFv derived from a monoclonal antibody against the α toxin of *Naja nigricollis* venom linked to *Escherichia coli* alkaline phosphatase in a bacterial system. However, they found that fusion of the scFv to alkaline phosphatase caused a moderate but significant reduction of the affinity of the scFv for its antigen. The catalysis rate of the fusion protein was not modified when compared with that of AChE extracted from venom (or control recombinant AChE), furthermore, the fusion protein is readily purified from culture supernatants of transfected COS cells by affinity chromatography using an AChE ligand.

The immunoconjugate produced in this study comprises a soluble monomeric form of AChE and an scFv. It was monovalent and had a molecular weight of less than 100 kDa. It has therefore a different structure than the alkaline phosphatase/anti-α-toxin scFv immunoconjugate synthesised by Ducancel et al. [14], which was bifunctional, containing a dimeric alkaline phosphatase associating two scFv molecules. In many circumstances, a monovalent immunoconjugate with a lower molecular weight should be more useful and more effective than a bivalent immunoconjugate of higher molecular weight. It should diffuse and bind to the antigen more rapidly and the presence of only one binding site per conjugate should prevent the formation of complexes when several antigen sites are present in a single structure. This reduces the risk of non-specific interactions.

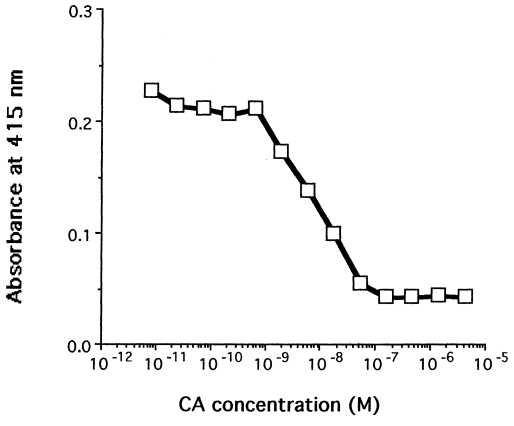

Fig. 3. Affinity of the fusion protein, AChE-scFv, for the CA subunit. Culture medium from COS cells transfected with the chimeric gene was diluted 1 in 800 and incubated overnight at 4°C with various concentrations of the CA subunit. AChE activity was measured using the method of Ellman.

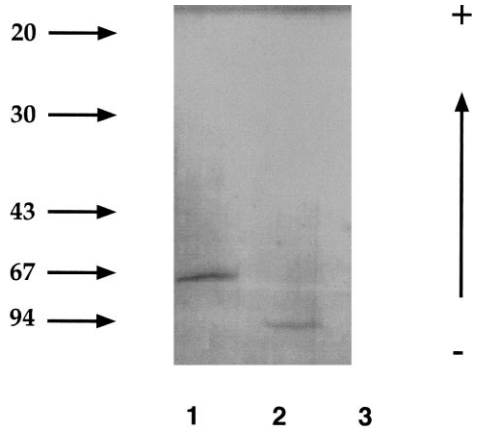

Fig. 4. Electrophoresis of the affinity-purified fusion protein. Denatured proteins in 1% SDS, 0.1 M β-mercaptoethanol were loaded onto a 12.5% polyacrylamide gel and subjected to electrophoresis. The gel was stained with Coomassie blue. The molecular weight markers are indicated by an arrow (phosphorylase b, 94 kDa; BSA, 67 kDa, ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa, trypsin inhibitor, 20 kDa); lane 1: AChE from *B. fasciatus* venom, purified by affinity chromatography; lane 2: AChE-scFv fusion protein purified by affinity chromatography.

The AChE of *B. fasciatus* is much more highly expressed than those of other species by transfected COS cells [15–19] and other eukaryotic expression systems [27]. The fusion protein was produced in large quantities and its concentration in the culture medium of transfected COS cells was about 1 μg/ml. The AChE-scFv immunoconjugate was produced and secreted by the COS cells in an active form that is readily and efficiently purified in a single step using cheap, reusable, affinity columns. This is more satisfactory than the procedure described by Ducancel et al. [14], where the alkaline phosphatase/scFv fusion protein precipitated in the cytoplasm of the bacteria and has to be renatured and purified by less efficient classical methods. However, their method nevertheless allowed the production of a useful recombinant tracer in significant amount, suggesting that even better results could be obtained with more efficient expression systems than COS cells, such as those involving yeasts or insect cells.

The method we have developed for the preparation of an immunoenzymatic tracer for detection of the CA subunit of crotoxin could be used for other molecules. It is based on the use of genetic engineering to produce a fusion protein containing the soluble, monomeric AChE from *B. fasciatus* and a scFv. This protocol could be adapted to the production of other immunoenzymatic tracers, so long as the specific scFv

required has been cloned using *Sfi*I and *Not*I sites. The *B. fasciatus* AChE in the N-terminal part of our fusion has very high activity, and this would presumably also be the case in any other analogous fusions. Moreover, it is straightforward to obtain highly pure preparations of this type of immunoenzymatic tracer by affinity chromatography. The production of conjugate could probably be improved by establishment of stable cell lines.

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