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Direct Detection of the Hydrolysis of Nerve Agent Model Compounds Using a Fluorescent Probe

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Abstract

Nerve agents are highly toxic organophosphorus compounds (OP) that are used as chemical warfare agents. Developing a catalytic bioscavenger to efficiently detoxify nerve agents in the bloodstream of affected individuals has been recognized as an attractive approach to prevent nerve agent toxicity. However, the search for nerve agent catalysts has been hindered by the lack of efficient direct assays for nerve agent hydrolysis. In addition, authentic nerve agents are restricted and access to use for experiments by the general research community is prohibited. Herein we report development of a method that combines use of novel nerve agent model compounds possessing a thiocholine leaving group that reacts with the fluorescent thio-detection probe, BES-Thio, to afford detection of sub-micromolar amounts of nerve agent model compounds hydrolysis products. The detection sensitivity of BES-Thio assay was approximately 10 times better than the Ellman assay. This developed method is useful as a direct, sensitive screening method for evaluating OP hydrolysis efficiency from catalytic cholinesterases. When the assay was assembled in the presence of oxime, OP-inhibited cholinesterases that were able to be reactivated by specific oxime showed oxime-assisted enzyme-mediated OP hydrolysis. Therefore, this method is also useful to screen oxime analogs to identify novel agents that can reactivate OP-inhibited cholinesterases or to screen various enzymes to identify pseudo-catalytic bioscavengers that can be readily reactivated by clinically approved oximes.

Keywords

nerve agent model compounds; OP hydrolysis; cholinesterase; fluorescent assay

1. Introduction

Nerve agents are highly toxic organophosphorus compounds (OP) that inhibit acetylcholinesterase (AChE). Serum cholinesterase, butyrylcholinesterase (BuChE), is a naturally occurring enzyme that scavenges stoichiometric amounts of nerve agents and protects AChE from low-level OP exposure. Developing a catalytic bioscavenger to efficiently detoxify nerve agents has been an attractive approach to prevent nerve agent toxicity [1,2]. However, such effort has been hindered by the lack of efficient direct assays

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for nerve agent hydrolysis. In addition, the use of authentic nerve agents by the general research community is restricted.

The ground-breaking work for identifying nerve agent hydrolytic BuChE variants came from the report on the BuChE variant G117H [3]. However, G117H BuChE hydrolyzes nerve agents slowly (e.g., turnover rate (k_{cat}) for sarin (GB) is 0.004 min^{-1} [3]). Due to technical difficulties to study this low amount of nerve agent hydrolyzed, indirect assays involving multiple steps of enzyme manipulation (e.g., inhibition, gel filtration, titration) have been adopted [3]. The procedures are time-consuming and cannot be easily adapted to a high throughput screening format.

We recently reported the chemical synthesis of a series of enantiomerically enriched nerve agent model compounds [4] by replacing the nerve agent leaving group (i.e., F^-, CN^- etc.) with thiocholine. These model compounds have been evaluated for cholinesterase inhibition potency and stereoselectivity as well as BuChE covalent adduct formation to validate their application as surrogates for authentic nerve agents [4,5]. Hydrolysis of the nerve agent model compound liberates a thiol that can be efficiently trapped by a fluorescent thiaphile such as BES-Thio. Herein we report the development of an efficient fluorescent assay that is capable of direct detection of low level hydrolysis of nerve agent model compounds. This approach compliments strategies previously reported, where OP analogs with larger fluorescent leaving groups were used as substrates [6,7]. The thiocholine leaving group for our analogs is the same as standard cholinesterase substrates acetylthiocholine and butyrylthiocholine (BTCh).

2. Materials and Methods

2.1. Materials

BES-Thio (2,4-dinitrobenzenesulfonyl, 2′,7′-dimethylfluorescein) was synthesized following procedures described previously [8]. The nerve agent model compounds, S_p-*O*-isopropyl *S*-(2-trimethylammoniumethyl) methylphosphonothioate iodide (S_pGBC) and S_p-*O*-cyclohexyl *S*-(2-trimethylammoniumethyl) methylphosphonothioate iodide (S_pGFC), were synthesized as previously described [4,9]. BTCh, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), pralidoxime chloride (2-PAM), and recombinant AChE were purchased from Sigma (St Louis, MO). Ecothiophate (ETP), highly purified human BuChE (serving as ELISA standards), and anti-BuChE polyclonal antibodies were gifts from Dr. Oksana Lockridge (University of Nebraska Medical Center, Omaha, NE). HI-6 was a gift from Dr. Douglas Cerasoli (USAMRICD, APG, MD). Recombinant WT and G117H BuChE used in the assay were expressed in CHO cells. Culture medium containing the secreted BuChE enzyme was concentrated using 50 kDa Amicon ultra centrifugal filters (Millipore, Bedford, MA) according to the manufacturer's protocol. The concentrated enzyme was washed 3 times with Tris-HCl buffer (40 mM, pH 7.4 at 25°C). Enzyme activity of the concentrated enzyme was determined by the Ellman assay [10]. Enzyme concentration was determined using ELISA. All other reagents and buffers used in this work were of analytical grade and commercially available.

2.2. BES-Thio assay detection of thiocholine

A thiocholine stock solution was prepared by hydrolyzing 5 mM BTCh with 0.5 M NaOH for 1 h followed by HCl neutralization. The concentration of thiocholine produced was confirmed with an Ellman assay. The thiocholine solution was serially diluted in 20 mM Tris-HCl buffer (pH 7.4 at 25 °C). BES-Thio stock solution (5 mM) was prepared in DMSO, and then diluted to 50 μM with isopropanol as a working solution. For the calibration curve of the BES-Thio assay, same volume of thiocholine (0.025–12.8 μM) and BES-Thio stock

solution in isopropanol was mixed in a flat bottom 96-well black plate (Greiner Bio-one, Frickenhausen, Germany) to achieve a final BES-thio concentration of 25 μM. The fluorescence signal was monitored for 20 min and data were collected every 1 min with a Victor^2 plate reader (Perkin Elmer, Waltham, MA) at an Ex/Em of 485nm/535nm. The relative fluorescent units (RFU) observed were plotted versus time after BES-Thio addition. The rate of fluorescent signal development (RFU/min) was calculated from the slope at the linear range of fluorescence development. Both the RFU at specific time points and RFU/min were plotted versus thiocholine concentration to construct standard curves. For comparison, the thiocholine calibration curve using the Ellman's reagent was done in parallel. The same amount of thiocholine (0.8 to 31.8 μM) and 100 μM DTNB was mixed in clear bottom 96-well plate (Greiner Bio-one). The absorbance was measured at 405 nm on the same plate reader.

For all the BES-Thio assays reported, OP hydrolysis incubations were terminated by addition of BES-Thio to achieve a final concentration of 25 μM and bring the final isopropanol amount to 50%. The fluorescence signal was monitored for 20 min and data were collected every 1 min as described above. A thiocholine standard series was prepared in parallel for all assays on the same assay plate as the samples. The slope of the fluorescence signal versus time plot for calibration standards was calculated and used to construct thiocholine calibration curves. Accordingly, the concentration of thiocholine was determined using these calibration curves for each hydrolysis incubation.

2.3. Hydrolysis of ETP by G117H BuChE

To evaluate the time-dependent hydrolysis of ETP by G117H BuChE, samples comprising of 0.43 μM G117H and 0.4 mM ETP were incubated in 20 mM Tris-HCl buffer (pH 7.4) in a 96-well plate format for various lengths of time (i.e., 5, 10, 20, 30, 45, 60, 90, 120, and 240 min) at room temperature. The formation of thiocholine from each incubation was determined with the BES-Thio method described above. The background fluorescence contributed to the overall signal from samples containing no substrate was subtracted.

To evaluate substrate-dependent ETP hydrolysis by G117H, 0.43 μM of G117H was incubated with ETP at various concentrations (i.e., 20, 40, 80, 140, 400, and 1000 μM) in 20 mM Tris-HCl buffer (pH 7.4) at room temperature for 1 h. Thiocholine production from each incubation was determined with the BES-Thio method described above. A plot of thiocholine producing rate versus ETP concentration afforded a hyperbolic curve that was fitted to the Michaelis-Menten equation to determine K_m and k_{cat}.

2.4. Hydrolysis of S_pGFC and S_pGBC by G117H BuChE

Hydrolysis of S_pGBC and S_pGFC by G117H BuChE was examined by incubating 0.4 mM nerve agent model compound with 0.43 μM G117H in 20 mM Tris-HCl buffer (pH 7.4) at room temperature for 4 h. Thiocholine production from each incubation was determined with the BES-Thio method described above. For comparison, single turnover of S_pGBC and S_pGFC by wild type BuChE was examined in parallel using the same assay.

2.5. Oxime-Assisted S_pGBC Hydrolysis by Wild Type AChE and BuChE

The oxime-assisted hydrolysis of S_pGBC by AChE or BuChE was examined using 2-PAM or HI-6, respectively. Enzyme (0.25 μM AChE or BuChE), 0.1 mM S_pGBC and 0.5 mM oxime (2-PAM or HI-6) were incubated in 20 mM Tris-HCl buffer solution (pH 7.4). At certain time points (i.e., 10, 30, 60, and 120 min), thiocholine production from each incubation was determined with the BES-Thio method described above.

2.6. Data analysis

All linear and non-linear regression analyses were done using Graphpad Prism Programs (Version 3.00, Graphpad, Inc., La Jolla, CA) to obtain the best fit values and standard errors. Z′ values were calculated to examine if the signal was reliably above background using a method previously reported [11].

3. Results and Discussion

3.1. Calibration curve evaluation for thiocholine detection

BES-Thio has previously been reported to show detection of glutathione, cysteine, and other free thiols in the picomole range [8]. For results described herein, detection of hydrolysis of nerve agent model substrates including S_pGFC, S_pGBC, and ETP (Figure 1) required monitoring the release of the thiocholine leaving group by forming a fluorescent product with BES-Thio (Figure 1). We first examined the chemical stability of thiocholine in the assay format described above (data not shown). Although not indefinitely stable, preparing fresh stock solutions of thiocholine and including parallel standard curves for all assays afforded reliable data. Because different free thiols have different reaction rates with BES-Thio [8], the incubation of thiocholine with BES-Thio was initially investigated to evaluate overall assay sensitivity. As shown in Figure 2A, the reaction of BES-Thio with thiocholine produced a linear increase in fluorescent signal for the initial few minutes and the slope of the fluorescent signal correlated with the concentration of thiocholine in each incubation.

RFU at 1 and 10 min were plotted versus thiocholine concentration (Figure 2B) and showed thiocholine-dependent fluorescence intensity. Z′ values were calculated from the signal at specific thiocholine concentration over background fluorescence levels [9]. Z′>0.5 was set as the threshold for the determination of the assay lower detection limit. The lower detection limit improved from 12.8 μM to 1.6 μM when the fluorescent signal was recorded at 10 min instead of 1 min after BES-Thio addition (Figure 2B). Linear regression analysis of the initial time-dependent increase in fluorescence intensity (i.e., 0–7 min) afforded the slope (i.e., RFU/min) as an indicator of fluorescent product formation rate (Figure 2A). A plot of each slope versus the thiocholine concentration showed an improved concentration-dependent linearity at the low thiocholine concentration range (i.e., R^2=0.995 for a thiocholine concentration between 0.2–12.8 μM) (Figure 2B). Using this method, the accuracy and precision of the assay improved as reflected in an increase in Z′ values (i.e., Z′ of 0.80–0.89 for slopes based analysis versus a Z′ value of 0.54–0.79 for a single time point based analysis (i.e.,10-min RFU) for thiocholine concentrations above 1.6 μM). The assay sensitivity was also improved to 0.4 μM (Z′=0.55) when calibration curves were generated based on slope. A comparison of standard curves obtained using the Ellman's reagent showed that the BES-Thio assay had ~10-fold greater detection sensitivity than the Ellman assay (data not shown). Thereafter, freshly prepared standard calibration curves were generated based on fluorescent product formation rate, RFU/min, for all the following assays and provided a sensitive means to quantify the hydrolysis of the target OP compounds with the BES-Thio method.

3.2. Hydrolysis of ETP by G117H BuChE

To explore the utility of the BES-Thio assay for OP hydrolysis detection, time-dependent ETP hydrolysis by catalytic BuChE variants G117H was examined. A plot of thiocholine formation versus incubation time showed a linear correlation during the first hour of incubation (Figure 3A). The rate of product formation became non-linear afterward, possibly due to a combination of factors including progressive aging of the ETP phosphonylated enzyme and/or loss of enzyme activity due to enzyme instability associated with extended incubation times. Substrate-dependent ETP hydrolysis was evaluated using a 1 h incubation

period and a G117H BuChE concentration of 0.43 μM. A fit of the product formation data to the Michaelis-Menten equation afforded a k_{cat} of 0.36 min^{-1} and a K_m of 42.7 μM (Figure 3B). These values are slightly lower than the reported value of k_{cat} 0.75 min^{-1} and K_m of 73 μM using purified G117H BuChE at 3 μM to examine ETP hydrolysis using a traditional Ellman assay [12]. The overall catalytic efficiency k_{cat}/K_m for ETP hydrolysis by G117H BuChE was comparable to the reported value (8.6 versus 10.3 $mM^{-1}min^{-1}$) [12].

3.3. Direct detection of nerve agent model compound hydrolysis by BuChE

The direct hydrolysis of GF and GB model compounds S_pGFC and S_pGBC by G117H BuChE was examined (Figure 4A). After 4 h incubation, thiocholine produced from S_pGFC and S_pGBC was 1.3 and 1.0 μM respectively, corresponding to 2–3 turnovers for each substrate. When wild type BuChE was examined under the same assay conditions, a stoichiometric amount of thiocholine was formed from an incubation of 0.43 μM WT BuChE with S_pGFC and S_pGBC, producing 0.45 and 0.42 μM thiocholine, respectively. Because the rate of S_pGFC and S_pGBC hydrolysis catalyzed by G117H was ~100-fold lower than the hydrolysis of ETP, the specific activity of enzyme-mediated hydrolysis could not be reliably estimated from these assays. However, based on the reported hydrolysis rate, a 4 h incubation of GB with G117H resulted in an estimated single turn-over (i.e., 0.24 h^{-1} × 4 h = 0.96) [3] plus the initial stoichiometric turnover based on phosphylation, the resultant estimated 2 turnover for G117H-mediated GB hydrolysis was in good agreement with the results reported herein for S_pGBC. Despite the limitations on the determination of specific activity for OP hydrolysis by G117H, the direct BES-Thio assay was useful to verify whether multiple turnover (i.e., hydrolysis) for OP substrates could occur for cholinesterase variants and provide a reliable means of ranking OP hydrolysis rates for multiple enzymes or enzyme variants in a high throughput format.

We also extended the analysis to oxime-assisted hydrolysis of S_pGBC by wild type AChE or BuChE. Traditionally, oxime-reactivation of OP-inhibited cholinesterase has been assayed through detection of regenerated cholinesterase activity following complete enzyme inhibition by excess OP inhibitors and then removal of excess OP inhibitors through gel filtration. The BES-Thio assay system described above was used to directly examine HI-6- and 2-PAM-mediated S_pGBC hydrolysis in a single step by both human wild type AChE and wild type BuChE, respectively. As shown in Figure 4B, HI-6 assisted hydrolysis of S_pGBC by WT AChE was most prominent. 2-PAM assisted S_pGBC hydrolysis by WT AChE and BuChE and HI-6 assisted hydrolysis by WT BuChE was detectably above background but relatively low. This finding is in good agreement with the previously reported oxime-mediated reactivation of sarin-inactivated AChE and BuChE [13].

4. Conclusions

The method reported herein involves the combined application of novel nerve agent model compounds possessing a thiocholine leaving group that can be combined with the fluorescent thio-detection probe, BES-Thio, to afford direct detection of sub micromolar hydrolysis of nerve agent model compounds. The method is useful as a sensitive high-throughput screening method for evaluating OP hydrolysis efficiency from cholinesterase variants or other OP catalytic enzymes. The assay can be used to efficiently identify oximes that can reactivate cholinesterases to afford net enzyme-mediated OP hydrolysis. The oxime-assisted assay can also be used for functional screening of enzyme variants readily reactivated by clinically approved oximes, thereby identifying pseudo-catalytic bioscavenger variants.

Acknowledgments

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Abbreviations

OP	organophosphorus compounds
AChE	acetylcholinesterase
BuChE	butyrylcholinesterase
ETP	echothiophate
GB	sarin
GF	cyclosarin
***S*$_p$GBC**	S_p-*O*-isopropyl *S*-(2-trimethylammoniumethyl) methylphosphonothioate iodide
***S*$_p$GFC**	S_p-*O*-cyclohexyl *S*-(2-trimethylammoniumethyl) methylphosphonothioate iodide
2-PAM	pralidoxime chloride
BES-Thio	2,4-dinitrobenzenesulfonyl fluorescein
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
BTCh	butyrylthiocholine iodide
RFU	relative fluorescent units

References

1. Lenz DE, Yeung D, Smith JR, Sweeney RE, Lumley LA, Cerasoli DM. Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: a mini review. Toxicology. 2007; 233(1–3): 31–39. [PubMed: 17188793]
2. Masson P, Nachon F, Broomfield CA, Lenz DE, Verdier L, Schopfer LM, Lockridge O. A collaborative endeavor to design cholinesterase-based catalytic scavengers against toxic organophosphorus esters. Chem Biol Interact. 2008; 175(1–3):273–280. [PubMed: 18508040]
3. Millard CB, Lockridge O, Broomfield CA. Design and expression of organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase. Biochemistry. 1995; 34(49):15925–15933. [PubMed: 8519749]
4. Barakat NH, Zheng X, Gilley CB, Macdonald M, Okolotowicz K, Cashman JR, Vyas S, Beck JM, Hadad CM, Zhang J. Chemical Synthesis of Two Series of Nerve Agent Model Compounds and Their Stereoselective Interaction with Human Acetylcholinesterase and Human Butyrylcholinesterase. Chem Res Toxicol. 2009 Epub ahead of print.
5. Gilley C, Macdonald M, Nachon F, Schopfer LM, Zhang J, Cashman JR, Lockridge O. Nerve Agent Analogues That Produce Authentic Soman, Sarin, Tabun, and Cyclohexyl Methylphosphonate-Modified Human Butyrylcholinesterase. Chem Res Toxicol. 2009 Epub ahead of print.
6. Amitai G, Adani R, Yacov G, Yishay S, Teitlboim S, Tveria L, Limanovich O, Kushnir M, Meshulam H. Asymmetric fluorogenic organophosphates for the development of active organophosphate hydrolases with reversed stereoselectivity. Toxicology. 2007; 233(1–3):187–198. [PubMed: 17129656]
7. Briseno-Roa L, Hill J, Notman S, Sellers D, Smith AP, Timperley CM, Wetherell J, Williams NH, Williams GR, Fersht AR, Griffiths AD. Analogues with fluorescent leaving groups for screening

and selection of enzymes that efficiently hydrolyze organophosphorus nerve agents. J Med Chem. 2006; 49(1):246–255. [PubMed: 16392809]

8. Maeda H, Matsuno H, Ushida M, Katayama K, Saeki K, Itoh N. 2,4-Dinitrobenzenesulfonyl fluoresceins as fluorescent alternatives to Ellman's reagent in thiol-quantification enzyme assays. Angew Chem Int Ed Engl. 2005; 44(19):2922–2925. [PubMed: 15818626]
9. Berman HA, Leonard K. Chiral reactions of acetylcholinesterase probed with enantiomeric methylphosphonothioates. Noncovalent determinants of enzyme chirality. J Biol Chem. 1989; 264(7):3942–3950. [PubMed: 2917983]
10. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7:88–95. [PubMed: 13726518]
11. Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen. 1999; 4(2):67–73. [PubMed: 10838414]
12. Lockridge O, Blong RM, Masson P, Froment MT, Millard CB, Broomfield CA. A single amino acid substitution, Gly117His, confers phosphotriesterase (organophosphorus acid anhydride hydrolase) activity on human butyrylcholinesterase. Biochemistry. 1997; 36(4):786–795. [PubMed: 9020776]
13. Worek F, Eyer P, Kiderlen D, Thiermann H, Szinicz L. Effect of human plasma on the reactivation of sarin-inhibited human erythrocyte acetylcholinesterase. Arch Toxicol. 2000; 74(1):21–26. [PubMed: 10817663]

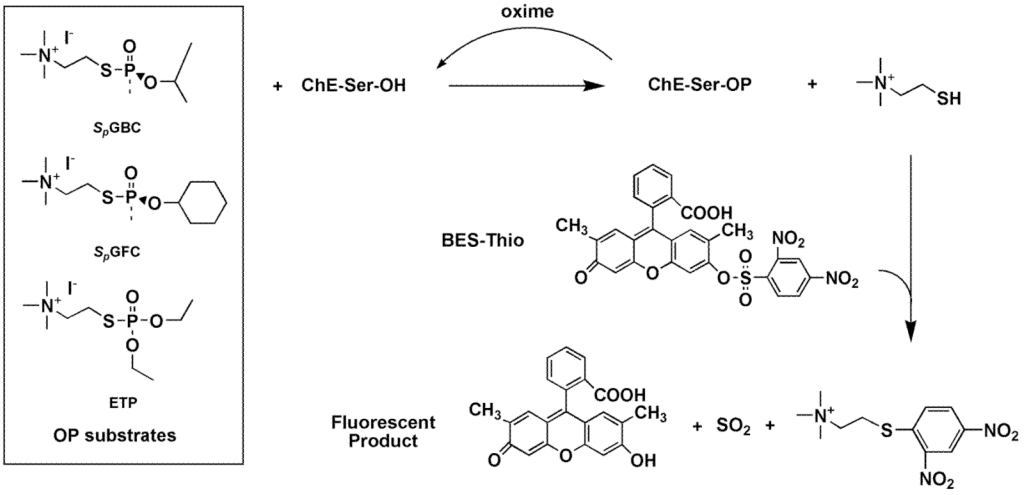


Figure 1.
Chemical reaction scheme of the Bes-Thio assay.

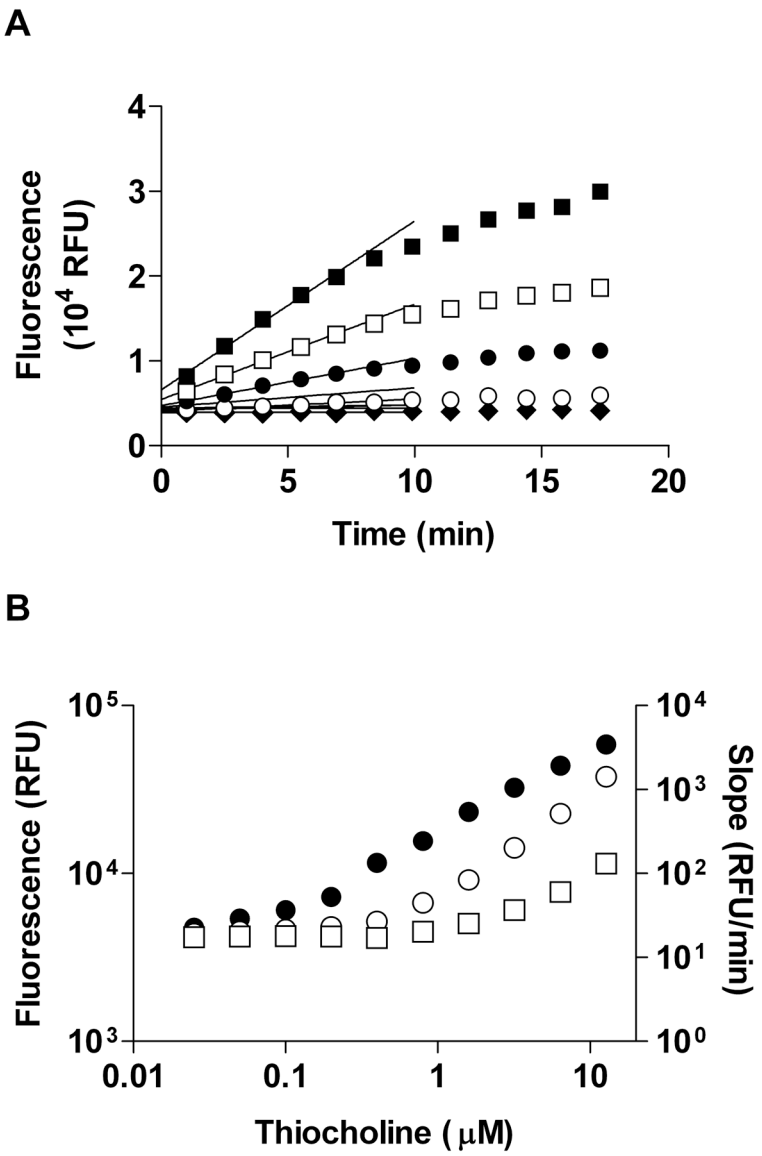
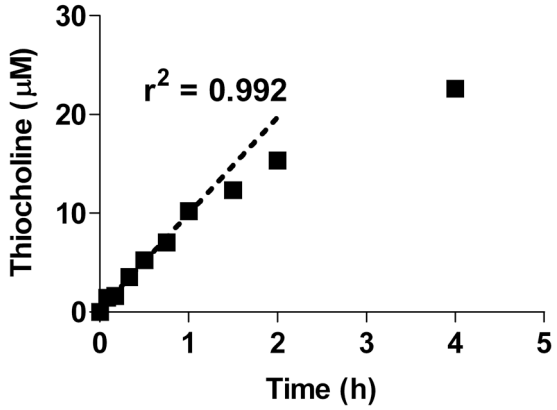


Figure 2.
(A). Time-dependent fluorescence intensity after incubating BES-Thio with representative concentrations of thiocholine: 0 μM (◆), 0.4 (○), 1.6 (●), 3.2 (□), and 6.4 μM (■). The lines show a linear fit of RFU data points observed between 0–7 min. Data points represent the average of triplicate samples and error bars are smaller than the data markers. (B) A replot of data shown from A for RFU values (left axis) observed at 1 min (□), 10 min (○), and the slope of RFU development calculated by the linear fit from Fig 2A (●, right axis) versus the thiocholine concentrations.



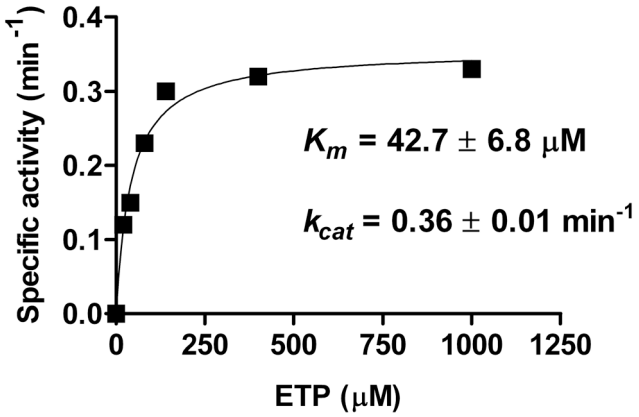


Figure 3.

(A) Time-dependent thiocholine formation from ETP hydrolysis by G117H BuChE. ETP (0.4 mM) was incubated with 0.43 μM G117H for various times at room temperature. Thiocholine formation was detected with the BES-Thio assay as described in the Methods. The dotted line represents the linear fit for incubation time for up to 1 h. (B) Substrate-dependent ETP hydrolysis by G117H BuChE. A number of concentrations of ETP was incubated with 0.43 μM G117H for 1h at room temperature. Thiocholine formation was detected by the BES-Thio assay as described in the Methods.

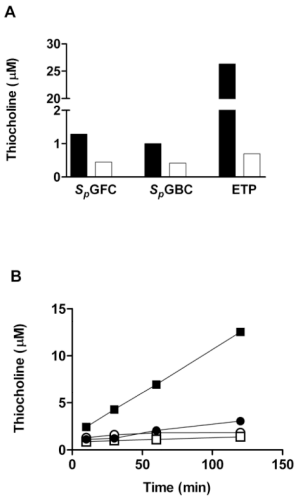


Figure 4.

(A) Thiocholine formation as detected by the BES-Thio method from the hydrolysis of different OPs (S_pGFC, S_pGBC and ETP) incubated with G117H (filled bars) or wild type BuChE (open bars) for 4 h. The OP concentration was 0.4 mM, and the enzyme concentration was 0.43 μM. (B) Thiocholine formation from oxime-mediated S_pGBC hydrolysis by wild type AChE (HI-6 (■) and 2-PAM (●) and wild type BuChE (HI-6 (□) and 2-PAM (○)) as detected by the BES-Thio method. In the incubation system, AChE or BuChE was 0.25 μM, S_pGBC was 0.1 mM, and oxime (2-PAM or HI-6) was 0.5 mM. The incubation times were 10, 30, 60 and 120 min, respectively.