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Optimal detection of cholinesterase activity in biological samples: Modifications to the standard Ellman's assay

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ABSTRACT

Ellman's assay is the most commonly used method to measure cholinesterase activity. It is cheap, fast, and reliable, but it has limitations when used for biological samples. The problems arise from 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), which is unstable, interacts with free sulfhydryl groups in the sample, and may affect cholinesterase activity. We report that DTNB is more stable in 0.09 M Hepes with 0.05 M sodium phosphate buffer than in 0.1 M sodium phosphate buffer, thereby notably reducing background. Using enzyme-linked immunosorbent assay (ELISA) to enrich tissue homogenates for cholinesterase while depleting the sample of sulfhydryl groups eliminates unwanted interactions with DTNB, making it possible to measure low cholinesterase activity in biological samples. To eliminate possible interference of DTNB with enzyme hydrolysis, we introduce a modification of the standard Ellman's assay. First, thioesters are hydrolyzed by cholinesterase to produce thiocholine in the absence of DTNB. Then, the reaction is stopped by a cholinesterase inhibitor and the produced thiocholine is revealed by DTNB and quantified at 412 nm. Indeed, this modification of Ellman's method increases butyrylcholinesterase activity by 20 to 25%. Moreover, high stability of thiocholine enables separation of the two reactions of the Ellman's method into two successive steps that may be convenient for some applications.

Cholinesterases (acetylcholinesterase [AChE][1] and butyrylcholinesterase [BChE]), the enzymes that cleave acetylcholine in the body, have been studied for 80 years. Results from studies with pure enzymes helped to characterize protein structure and enzymatic behavior in the presence of different substrates and/or inhibitors.

Recent observations, however, underline the importance of studying the native enzyme in its biological environment as cholinesterases have been linked to etiopathogenesis of some diseases such as cancer [1,2], Alzheimer's disease [3–7], Parkinson's diseases [8,9], cardiovascular diseases, and obesity [10–15]. Moreover, cholinesterases are principal targets of nerve agents, pesticides, and drugs used to prevent muscle weakness in myasthenia gravis or to reduce loss of memory in patients with Alzheimer's disease.

An efficient method to examine cholinesterase activity in biological samples, therefore, is essential. Available methods, however, do not embrace factors in the biological environment, that is, the presence of other molecules that could interfere with the assays and low cholinesterase activities in some tissues that may be at the limit or under the limit of detection.

The most commonly used method to study cholinesterase activity is an assay described by Ellman and coworkers [16] with more than 12,900 citations of the original article as reported by the Web of Science. Ellman's method is a two-reaction assay (Fig. 1A). In the first reaction, cholinesterase (AChE or BChE) hydrolyzes a thioester into an intermediate thiocholine (TCh). In the second reaction, TCh interacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), giving a yellow product, 2-nitro-5-thiobenzoic acid (TNB). The intensity of the color is proportional to the level of the hydrolysis product, TCh.

The method is fast, accurate, and inexpensive, and it enables kinetic analysis of the enzymatic reaction. Despite its advantages, it has limitations that are especially important when used for

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[1] *Abbreviations used:* AChE, acetylcholinesterase; BChE, butyrylcholinesterase; TCh, thiocholine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoic acid; EA, Ellman's assay; SH, sulfhydryl; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; BTC, butyrylthiocholine iodide; ATC, acetylthiocholine iodide; BW, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; iso-OMPA, tetraisopropyl pyrophosphoramide; 2s–EA, two-step Ellman's assay; ITC, isothermal titration calorimetry.

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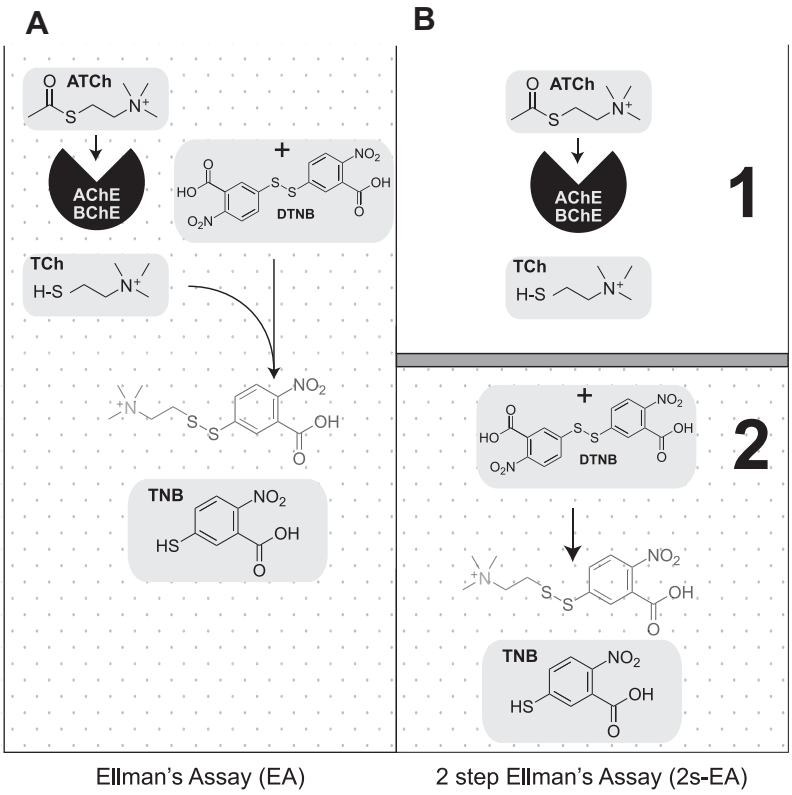

Fig.1. (A) Principle of standard Ellman's assay (EA). Cholinesterase (AChE or BChE) hydrolyzes thioester (here shown for acetylthiocholine, ATC) into an intermediate thiocholine (TCh) that interacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The color intensity given by the 2-nitro-5-thiobenzoic acid (TNB) product is proportional to the level of produced TCh. (B) Principle of modified two-step Ellman's assay (2s–EA). Substrate (here shown with ATC) is hydrolyzed into TCh by cholinesterase in the absence of DTNB in the mixture (1). In the second step (2), time-dependent production of TCh is revealed by adding DTNB at different times, and OD_{412} is measured immediately. If needed, enzyme may be inhibited after the first step and the second step may be performed with delay.

biological samples and especially when low activities are being followed (due to the low level of cholinesterases or due to the inhibition of the enzyme). The most important limiting factors of the Ellman's assay (EA) are the sensitivity of DTNB to light, instability of the reagent solution over time, high background in biological samples due to the interaction with abundant free sulfhydryl (SH) groups, and low detection sensitivity that does not allow detecting low cholinesterase activities. Over the past few decades, a few modifications were introduced in order to improve the method; however, most were focused on specific conditions (for a review, see Ref. [17]), for example, multi-sample assay [18–20], whole-blood screening [21–25], and OP and other inhibitor screening [26–28]. The method of Johnson and Russell [29], based on the hydrolysis of radioactive acetylcholine, is an efficient alternative that solves the problems of the original Ellman's method in biological samples. It provides high sensitivity and low background. However, the manipulation of radioactivity is limited in numerous countries and laboratories.

Here we present optimization of EA to study cholinesterases in biological sample even at low activity levels. We document the stability of the reagents and products in Hepes buffer. Because TCh appears to be very stable, we proposed to split the two reactions of the Ellman's method into two sequential steps. This modification of the assay solves several problems due to DTNB. Moreover, it reveals for the first time an interference of DTNB with BChE activity that can cause a misinterpretation of the results when low BChE activity is followed over a prolonged time period. To solve the problem of the nonspecific interaction of DTNB in biological samples, we propose the use of enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Pure enzymes

Pure enzyme solutions and biological samples were used in the study. Purified recombinant human full-length BChE was a gift from O. Lockridge (Eppley Institute, University of Nebraska Medical Center [UNMC], Omaha, NE, USA). The enzyme was produced for crystallographic analysis, and it is partially deglycosylated [30]. Purified native human BChE was purified from plasma and was a gift from D. Lenz (U.S. Army Medical Research Institute of Chemical Defense [USAMRICD], Aberdeen Proving Ground, MD, USA). Pure recombinant mouse BChE was a gift from A. Saxena (Walter Reed Army Institute of Research [WRAIR], Silver Spring, MD, USA). Pure recombinant mouse AChE was a gift from P. Taylor (University of California, San Diego, La Jolla, CA, USA).

Plasma preparation

Plasma from human, mouse, rat, and dog were used. Venous blood was collected into ethylenediaminetetraacetic acid (EDTA)- or heparin- treated collection tubes (S-Monovette® EDTA K2 Gel, Sarstedt, product no. 04.1931, or Microvette® 200 LH, Sarstedt, product no. 20.1292) and centrifuged at 14,000*g* for 10 min at 4 °C. Supernatant containing plasma was flash frozen in liquid nitrogen and stored at −80 °C until use.

Tissue extraction

Mice were anesthetized with chloral hydrate, perfused transcardially with ice-cold physiological saline, and euthanized. Brain