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Heat pretreatment eliminates spurious butyrylcholinesterase enhancement of endotoxin levels in the kinetic chromogenic assay

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ABSTRACT

The kinetic chromogenic endotoxin assay measures the release of p-nitroaniline from the chromogenic peptide substrate Ac-IEAR-pNA. As part of our project to purify large quantities of human butyrylcholinesterase (HuBChE), we evaluated pure HuBChE for endotoxin levels. We found that HuBChE contributed up to 90% of the yellow p-nitroaniline product in a standard endotoxin assay through the catalytic hydrolysis of Ac-IEAR-pNA with a rate constant of 0.016 min^{-1} and a K_m of 2.9 mM in potassium phosphate buffer pH 7.0 at 24 °C. Thus, endotoxin concentrations for native BChE are artificially high in the kinetic chromogenic assay. Destruction of HuBChE catalytic activity by boiling yields endotoxin concentrations that more accurately reflect the endotoxin concentration in purified HuBChE preparations.

1. Introduction

Endotoxins are lipopolysaccharides derived from the cell wall of gram-negative bacteria such as *Escherichia coli, Salmonella, and Pseudomonas*. Endotoxins injected into animals or humans can cause a systemic inflammatory response, multiple organ failure, septic shock, disseminated intravascular coagulation, and death [1]. Products intended for injection into humans must not deliver more than 5.0 endotoxin units/kg body weight per hour [2]. Rodents can tolerate much higher endotoxin levels than humans [3]. We manufacture pure human butyrylcholinesterase (HuBChE) for use as a nerve agent bioscavenger in animal studies. We measure endotoxin levels in our HuBChE preparations using the kinetic chromogenic assay from Charles River Laboratories.

The Ac-IEAR-pNA peptide is the chromogenic substrate for quantifying endotoxin levels. The kinetic chromogenic endotoxin assay uses a bacterial endotoxin to activate a protease in Limulus Amebocyte Lysate (LAL) that hydrolyzes the colorless substrate Ac-Ile-Glu-Ala-Arg-pNA, resulting in the production of p-nitroaniline. The time to appearance of the yellow p-nitroaniline product is proportional to the concentration of endotoxin in a sample [4,5].

HuBChE has aryl acylamidase activity, splitting 2-nitroaniline from 2-nitroacetanilide (2-NAA) to produce the yellow 2-nitroaniline product as shown in Fig. 1 [6–9]. Other acetanilides including 3-(acetamido)N,N,N-trimethylanilinium and o-nitrotrifluoroacetanilide are also hydrolyzed by HuBChE [7,8].

While testing endotoxin levels in highly concentrated HuBChE samples, we questioned whether the acylamidase activity of HuBChE contributed to the apparent endotoxin level in our preparations. If both HuBChE and the endotoxin-activated LAL hydrolyzed Ac-IEAR-pNA, then the apparent endotoxin concentration in purified HuBChE preparations would be artificially high.

2. Materials and methods

2.1. Reagents and materials

The Endochrome-K kit was used for the kinetic chromogenic LAL assay of endotoxin levels (Charles River cat# R1708K). This kit contains the *E. coli* strain 055:B5 endotoxin standard, LAL reagent water, and lyophilized LAL with chromogenic substrate. Additional

Abbreviations: HuBChE, human butyrylcholinesterase; DMSO, dimethylsulfoxide; BTC, butyrylthiocholine; 2-NAA, 2-nitroacetanilide; Ac-IEAR-pNA, Ac-Ile-Glu-Ala-Arg-pNitroacetanilide; LAL, limulus amebocyte lysate; EU/mL, endotoxin units per mL.

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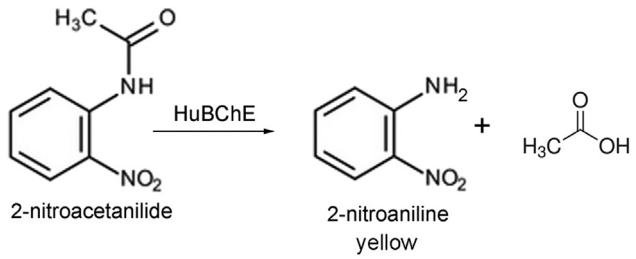

Fig. 1. Aryl acylamidase activity of HuBChE.

LAL was purchased from Charles River (catalog # R1710K) as needed. The chromogenic substrate Ac-IEAR-pNA peptide (MW 649.7) was obtained from bioWorld (Dublin, OH, catalog # 21970008-1) and 2-nitroacetanilide was obtained from Lancaster Synthesis Inc. (Pelham, NH, catalog # 16278). Human BChE (accession # P06276) was purified from Cohn fraction IV-4 by the authors. Hupresin is a new affinity gel for purification of BChE [10]. Hupresin was synthesized by Emilie David at the University of Rouen in France (emilie.david@insa-rouen.fr). The ligand is a small, custom synthesized molecule called huprine 19. The affinity gel was produced by coupling the amine group of huprine 19 to carboxyl groups in ECH-Sepharose 4B (GE Healthcare, catalog # 17-0571) using carbodiimide. The ECH-Sepharose 4B has a spacer arm of 6-aminohexanoic acid at a density of 12–16 μmoles carboxyl groups/mL of drained gel.

2.2. Human butyrylcholinesterase

Plasma-derived HuBChE was purified from Cohn fraction IV-4 paste by anion exchange chromatography at pH 4.5, followed by affinity chromatography on a hupresin affinity gel [10]. The highly purified HuBChE consisted of tetramers with a molecular weight of 340,000 Da. The protein concentration of HuBChE was calculated from its activity with butyrylthiocholine, using a specific activity of 500 units/mg for BChE purified from frozen Cohn paste.

Activity assays were performed after serially diluting HuBChE into 1 mg/mL human albumin in phosphate buffered saline to an activity of 5–10 units/mL. HuBChE stock solutions diluted with 1 mg/mL albumin had 10% higher activity compared to HuBChE diluted in buffer without albumin. Albumin minimized adsorption of HuBChE to the walls of the tubes. The activity of a 10 μL aliquot of the diluted HuBChE was assessed in 2 mL of 0.1 M potassium phosphate pH 7.0 containing 1.0 mM butyrylthiocholine iodide and 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) at 25 °C. The increase in absorbance at 412 nm was recorded in a Gilford spectrophotometer interfaced to MacLab. The slope for the time interval 10–40 s was used to calculate activity using the extinction coefficient 13,600 M^{-1} cm^{-1} [11]. Units of activity are defined as μmoles butyrylthiocholine hydrolyzed per min.

2.3. Determination of K_m and k_{cat} values for Ac-IEAR-pNA

A 16 mM stock solution of Ac-IEAR-pNA was prepared by dissolving 0.014 g in 1.35 mL of 0.05 M potassium phosphate pH 7.0. Wells in a 96-well microplate received 90 μL of Ac-IEAR-pNA at different dilutions in buffer and 10 μL BChE so that the final Ac-IEAR-pNA concentrations ranged from 1 to 12 mM in a total volume of 100 μL. The BChE stock solution with an activity of 75,000 units/mL (150 mg/mL) delivered 17.6×10^{-9} mol of BChE active sites per well. The absorbance increase at 415 nm was recorded in a BioTek Synergy H1 Hybrid microplate reader for 60 min at 24 °C. The rate of hydrolysis of Ac-IEAR-pNA at 415 nm was calculated using the extinction coefficient 8000 M^{-1} cm^{-1} for p-nitroaniline [12] and a pathlength of 0.3 cm for 100 μL sample in a 96-well plate.

2.4. Determination of K_m and k_{cat} values for 2-nitroacetanilide

A 0.75 M stock solution of 2-NAA was prepared by dissolving 0.027 g of 2-NAA in 0.2 mL dimethylsulfoxide (DMSO). The solution was diluted to 9.36 mL with 0.05 M potassium phosphate pH 7.0 to make a 16 mM stock solution containing 2% DMSO. The 16 mM stock solution was further diluted with 0.05 M potassium phosphate pH 7.0 containing 2% DMSO to make 2-NAA solutions ranging in concentration from 2 to 15.2 mM. Each well in a 96-well plate received 190 μL of 2-NAA and 10 μL of BChE (550 units/mL in phosphate buffered saline and 1.5 mM sodium azide) so that the quantity of BChE active sites in each well was 0.13×10^{-9} mol (5.5 units of activity). The absorbance increase at 430 nm was recorded in a BioTek Synergy H1 hybrid microplate reader for 10 min at 24 °C. The rate of hydrolysis of 2-NAA at 430 nm was calculated using the extinction coefficient 4000 M^{-1} cm^{-1} for 2-nitroaniline [13] and a pathlength of 0.5 cm for 200 μL sample in a 96-well plate.

2.5. Endotoxin levels in native and heat-denatured HuBChE

A pure HuBChE preparation in 52 mM L-His pH 6.0 with an activity of 20,000 units/mL (40 mg/mL) was serially diluted with endotoxin-free water to evaluate the effect of BChE amidase activity on the apparent endotoxin levels in the kinetic chromogenic LAL assay. A second set of serially diluted samples was prepared from heat-denatured HuBChE that had lost all activity with the substrate butyrylthiocholine. The heat-denatured HuBChE samples were prepared by heating a 0.5 mL aliquot of HuBChE (40 mg/mL) in a boiling water bath for 3 min and serially diluting the denatured HuBChE with LAL water. Wells of a sterile 96-well plate received 100 μL of HuBChE. The hydrolysis reactions were initiated by adding 100 μL of the LAL reagent, which contained the chromogenic substrate, to each well after the temperature in the BioTek Synergy H1 hybrid microplate reader had reached 37 °C. The increase in absorbance at 405 nm was recorded for 1.5 h. Serial dilutions of the endotoxin standard yielded a standard curve for calculating the endotoxin concentrations in the BChE samples. Data were calculated with Gen5 2.01 Data Analysis Software. The assay is based on the time it takes to see an absorbance increase of 0.2 at 405 nm. The study was repeated four times for a total of 16 replicates for each BChE dilution.

3. Results

3.1. Hydrolysis of Ac-IEAR-pNA peptide by HuBChE

The chromogenic substrate Ac-IEAR-pNA was cleaved by HuBChE to produce the yellow p-nitroaniline product detected at 415 nm indicated in Fig. 2. A control reaction containing 1 mM chromogenic peptide, but no HuBChE, showed no change in absorbance during the 1 h measurement period. This result demonstrated that HuBChE hydrolyzes the amide bond that links p-nitroaniline to arginine in the peptide Ac-IleGluAlaArg-pNA.

3.2. K_m and k_{cat} values for Ac-IEAR-pNA hydrolysis by HuBChE

The rate of hydrolysis of the chromogenic substrate increased 3-fold when the concentration of Ac-IEAR-pNA was increased from 1 mM to 8 mM, as shown in Fig. 3A. No further increase in rate was observed with Ac-IEAR-pNA concentrations higher than 8 mM.

The Lineweaver-Burk plot in Fig. 3B yielded a K_m value of 2.9 mM and a k_{cat} value of 0.016 min^{-1} for the hydrolysis of Ac-