Characterization of Butyrylcholinesterase from Porcine Milk

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Abstract

Human butyrylcholinesterase (HuBChE) is under development for use as a pretreatment antidote against nerve agent toxicity. Animals are used to evaluate the efficacy of HuBChE for protection against organophosphorus nerve agents. Pharmacokinetic studies of HuBChE in minipigs showed a mean residence time of 267 h, similar to the half-life of HuBChE in humans, suggesting a high degree of similarity between BChE from 2 sources. Our aim was to compare the biochemical properties of PoBChE purified from porcine milk to HuBChE purified from human plasma. PoBChE hydrolyzed acetylthiocholine slightly faster than butyrylthiocholine, but was sensitive to BChE-specific inhibitors. PoBChE was 50-fold less sensitive to inhibition by DFP than HuBChE and 5-fold slower to reactivate in the presence of 2-PAM. The amino acid sequence of PoBChE determined by liquid chromatography tandem mass spectrometry was 91% identical to HuBChE. Monoclonal antibodies 11D8, mAb2, and 3E8 (HAH 002) recognized both PoBChE and HuBChE. Assembly of 4 identical subunits into tetramers occurred by noncovalent interaction with polyproline-rich peptides in PoBChE as well as in HuBChE, though the set of polyproline-rich peptides in milk-derived PoBChE was different from the set in plasma-derived HuBChE tetramers. It was concluded that the esterase isolated from porcine milk is PoBChE.

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All authors contributed data, prepared figures and tables, and wrote parts of the manuscript. All authors have given approval to the final version of the manuscript.

Conflict of Interest
The authors declare no competing financial interest.

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Keywords

butyrylcholinesterase; acetylcholinesterase; porcine; polyproline; tetramer; Hupresin

1. Introduction

Vertebrates have two types of cholinesterase (ChE) - acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BChE, E.C.3.1.1.8). AChE is present in cholinergic synapses in the brain, in autonomic ganglia in the neuromuscular junction, and in the target tissues of the parasympathetic system; its major function is to terminate neurotransmission. Human BChE (P06276) on chromosome 3q26 and human AChE (P22303) on chromosome 7q22 share 70% sequence similarity and 52% sequence identity. Though BChE is widely distributed in organs and tissues, people with a hereditary absence of BChE have no symptoms, making it difficult to assign a physiological function to BChE. BChE is primarily synthesized in the liver and is secreted into the plasma. It has been suggested that BChE in plasma functions as a bioscavenger, thereby protecting AChE from inactivation by naturally occurring toxins. This role of BChE is supported by many studies, which demonstrate that exogenously administered BChE can provide protection from the toxicity of organophosphorous (OP) nerve agents. The involvement of brain BChE in neurotransmission has been demonstrated. Each enzyme can be distinguished from the other on the basis of substrate specificity and sensitivity to various inhibitors. Although AChE is most efficient at hydrolyzing acetylcholine, BChE exhibits less substrate specificity and efficiently hydrolyzes butyryl-, propionyl-, acetyl-, and benzoyl-choline. The two enzymes also can be distinguished by their sensitivity to inhibition by AChE-specific inhibitors BW284c51 and huperzine A, and BChE-specific inhibitors ethopropazine and iso-OMPA.

BChE purified from human plasma (HuBChE) has been studied extensively due to its therapeutic applications. Exogenously administered HuBChE can counteract the toxicity of OP nerve agents and pesticides, detoxify cocaine, and alleviate succinylcholine-induced apnea. Our laboratory has focused on developing HuBChE as a bioscavenger for the prophylaxis of OP nerve agent toxicity in humans. For ethical reasons, the efficacy of HuBChE cannot be investigated in humans. Therefore, the toxicity of OP nerve agents and the efficacy of HuBChE against multiple LD50 doses of OP nerve agents are evaluated in animal models. Results from animal studies are extrapolated to humans. Due to many similarities in anatomy and physiology to humans, pigs including minipigs are used for evaluating toxicity from percutaneous, intramuscular, intravenous, and inhalation exposure to OP nerve agents. Pigs have also been used to evaluate protection against OP nerve agent toxicity afforded by pretreatment with bioscavengers such as HuBChE. Pharmacokinetic studies showed that the mean residence time of plasma-derived, tetrameric HuBChE in minipigs was 267 h (data not shown), indicating that HuBChE was not rapidly cleared from the circulation of pigs, but remained in the circulation with a half-life similar to that in humans. This suggests a high degree of similarity between human (Hu) and porcine (Po) BChE.
Although the biochemical properties of HuBChE are well-characterized, the properties of PoBChE are largely unknown. A choline ester hydrolyzing enzyme, partially purified from porcine milk and porcine parotid gland was identified as PoBChE based on substrate and inhibitor specificity. In agreement with Augustinsson and Olsson we found that porcine milk was a richer source of BChE than porcine plasma. BChE activity was 1–3 U/mL in milk and 0.2 U/mL in plasma, where Units of activity are μmoles butyrylthiocholine hydrolyzed per min. Therefore milk was chosen as the source of PoBChE for the current studies. PoBChE was purified using high speed centrifugation followed by procainamide affinity and gel permeation chromatography. A side-fraction was purified by Hupresin affinity chromatography. The catalytic and inhibitory properties of PoBChE were compared with those of HuBChE and recombinant human acetylcholinesterase (rHuAChE). The bioscavenging properties were evaluated by comparing inhibition by diisopropyl fluorophosphate (DFP) and the aging and reactivation of DFP-inhibited enzyme. The amino acid sequence of PoBChE and the identity of polyproline-rich peptides embedded in PoBChE tetramers were determined by mass spectrometry analysis. Enzyme-linked immunosorbent assays identified 3 anti-HuBChE monoclonal antibodies that recognize PoBChE.

2. MATERIALS AND METHODS

2.1. Materials

HuBChE was purified from Cohn fraction IV-4 paste, as described. Recombinant human acetylcholinesterase (rHuAChE) expressed in CHO cells and purified using procainamide Sepharose 4B affinity chromatography, was provided by Dr. Nageswararao Chilukuri (Division of Biochemistry, Walter Reed Army Institute of Research). Porcine milk was from Waltz farms (Hagerstown, MD). (7-(O,O-diethyl-phosphinyloxy)-1-methylquinolinium methylsulfate (DEPQ) was provided by Drs. Yacov Ashani and Haim Leader (Israel Institute for Biological Research, Ness-Ziona, Israel). Pyridine-2-aldoxime methiodide (2-PAM) was obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. Bio-Spin® 6 chromatography columns and Biogel A 1.5 m column were from Bio-Rad Laboratories (Hercules, CA). YMC-Pack Diol-300 column for HPLC was from Waters Corp. (Milford, MA). Procainamide Sepharose 4B was from Sigma Chemical Co. (St. Louis, MO). Hupresin Sepharose 4B was synthesized by Emilie David at CHEMFORASE, 76130 Mont Saint-Aignan, France. Mouse anti-HuBChE monoclonal antibodies 11D8 (accession KT189147 and KT189148), mAb2 (accession KJ141199 and KJ141200), and B2 18–5 (accession KT189143 and KT189144) are described. The commercially available mouse anti-HuBChE monoclonal antibody 3E8 (HAH 002–01) was from BioPorto Diagnostics, Denmark via Antibody Shop. All reagent grade chemicals including acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3 (BW284c51), decamethonium bromide, edrophonium chloride, ethopropazine hydrochloride, tetrakispropyl pyrophosphoramide (iso-OMPA), propidium iodide, tacrine, N-[tris(hydroxymethyl)methyl]-3-amino propanesulfonic acid (TAPS), sodium phosphate, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO).
2.2. Isolation and Purification of Cholinesterase from Porcine Milk

Porcine milk (1200 mL) was defatted by centrifugation at 5,200 × g for 20 min at 4 °C. The purification of BChE from defatted milk was conducted essentially as described. Defatted milk was combined with 25 mL of procainamide-Sepharose 4B affinity gel and stirred overnight at 4 °C. The gel was washed with 500 mL of 50 mM sodium phosphate, pH 8.0, and packed into a 1.5 cm x 20 cm column. Bound PoBChE was eluted with 0.1 M procainamide in 50 mM sodium phosphate, pH 8.0. Fractions containing BChE activity were pooled and dialyzed against 10 mM sodium phosphate, pH 8.0. The enzyme was loaded onto a 1 cm x 20 cm column packed with 10 mL of procainamide-Sepharose 4B gel, washed until the A280 of the effluent was <0.01 and eluted with 0.1 M procainamide in 50 mM sodium phosphate, pH 8.0. Fractions containing BChE activity were pooled, concentrated in an Amicon stirred cell with a PLGC 30 membrane (Amicon, Beverly, MA) and loaded onto a Biogel A 1.5 m column (1.5 cm x 170 cm) equilibrated in 50 mM sodium phosphate, pH 8.0. Fractions containing BChE activity were pooled, concentrated using an Amicon PLGC 30 membrane, and stored in 50 % glycerol at −20 °C. Protein concentration was calculated from absorbance at 280 nm where an absorbance of 1.8 corresponded to 1 mg/mL.

A side-fraction of partially purified PoBChE that had been stored in 50% glycerol at −20°C for more than 5 years was purified by affinity chromatography on Hupresin. The PoBChE was dialyzed against 20 mM Tris.HCl pH 7.5 to remove glycerol. A total of 174 units in 13.6 mL were loaded at room temperature onto 16 mL of Hupresin packed in a Pharmacia C16/20 column. Contaminating proteins were washed off with 120 mL of 20 mM Tris.HCl pH 7.5, 0.05% azide followed by 90 mL of 0.3 M NaCl in 20 mM Tris.HCl pH 7.5, 0.05% azide. PoBChE was eluted with 60 mL of 0.1 M tetramethylammonium bromide in 20 mM Tris.HCl pH 7.5, 0.05% azide, with a recovery of 100 units. An additional 40 units were recovered by washing the column with 40 mL of 2 M NaCl in buffer. Proteins were concentrated and desalted in Centricon YM-30 (Millipore/Amicon cat no 4209) centrifugal filters. A portion of the Hupresin-purified PoBChE was used for SDS gel electrophoresis. Another portion was used for LC-MS/MS to identify polyproline-rich peptides and to determine the amino acid sequence of PoBChE.

2.3. Measurement of Cholinesterase Activity

AChE or BChE hydrolysis of acetylthiocholine (ATC), propionylthiocholine (PTC), or butyrylthiocholine (BTC) was measured in 50 mM sodium phosphate buffer, pH 8.0, at 22 °C in the presence of 1.0 mM 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), as described. To determine the catalytic parameters for AChE or BChE, initial velocity was measured as a function of ATC, BTC, or PTC over a concentration range of 0.01 – 28 mM, using a microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA). Units of activity are µmoles per min.

2.4. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Hupresin-purified PoBChE was deglycosylated with PNGaseF, reduced with 10 mM dithiothreitol, alkylated with 50 mM iodoacetamide, digested with trypsin, and analyzed by LC-MS/MS on the 6600 Triple-TOF mass spectrometer (AB Sciex, Framingham, MA). Details of the protocol have been described. Tryptic peptides were searched against the
NCBI n 15Sep2014 database for *Sus scrofa* proteins using the Paragon algorithm from Protein Pilot v5.0.1 (AB Sciex, Framingham, MA). Polyproline-rich peptides were searched using the following parameters: protease none, species *Sus scrofa*, database NCBI n 15SEP2014.fasta.

The *Sus scrofa* proteins of interest to this work have been deleted from the NCBI n 2016.5.30 database, but are available in the NCBI n 2013.6.17 and 2015.3.10 databases where the accession numbers for PoBChE are gi335299867 and XP_003358712. Based on our mass spectrometry results, NCBI has assigned accession number NP_001344438.1 to PoBChE in a file dated 04 Nov 2017.

2.5. **Enzyme-Linked Immunosorbent Assay (ELISA)**

Immulon 4HBX flat bottom 96-well polystyrene plates (Thermo Scientific 3855) were coated with 1 μg of goat anti-mouse IgG (Sigma M8642) in 100 μL PBS at 4°C overnight. Wells were blocked with 200 μL of 1% bovine serum albumin (BSA) in 20 mM Tris.HCl pH 7.4, 0.15 M NaCl (Tris-buffered saline, TBS) for 1 h, washed once with TBS containing 0.05% Tween-20 (TBST), and incubated with 1 μg/100 μL of anti-human monoclonal antibodies 11D8, mAb2, 3E8, or B2 18–5 for 2 h. Wells were washed 3 times with TBST. PoBChE was diluted with 1 mg/mL BSA in TBS to make 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 ng PoBChE/100 μL Each diluted PoBChE solution was added to 8 replicate wells on 4 plates. Control wells were treated with 1 mg/mL BSA in TBS (no PoBChE). Plates were incubated for 1 h, then washed 3 times with TBST. Bound PoBChE was quantified by addition of 100 μL of 1 mM BTC in 0.1 M potassium phosphate pH 7.0 containing 0.5 mM DTNB. The yellow color that developed was recorded at 405 nm in a BioTek plate reader (Winooski, VT).

2.6. **Determination of Active-Site Concentration**

The concentration of active sites in rHuAChE and PoBChE was determined by titration with 7-(O,O-diethyl-phosphinyloxy)-1-methylquinolinium methylsulfate (DEPQ) as described 27

2.7. **Analysis of Catalytic Parameters**

The catalytic parameters $K_m$, $K_{ss}$, and $b$, were obtained by non-linear, least squares fitting of the data for the hydrolysis of ATC, BTC, or PTC, to Equation 1 28, using GraphPad Prism (v3.0; GraphPad Software Inc., San Diego, CA):

$$v = \left( \frac{1 + b[S]/K_{ss}}{1 + [S]/K_{ss}} \right) \frac{V_{max}}{1 + Km/[S]}$$  \text{ Equation 1}

where, $v$ is the initial velocity, $V_{max}$ is the maximal velocity, $[S]$ is the concentration of substrate, $K_m$ is the Michaelis-Menten constant, $K_{ss}$ is the dissociation constant for excess substrate activation/inhibition, and $b$ reflects the efficiency of hydrolysis by the ternary enzyme substrate complex. When $b = 1$, the enzyme follows Michaelis-Menten kinetics, whereas substrate inhibition (as in AChE) is indicated when $b < 1$, and substrate activation (as in BChE) is indicated when $b > 1$.
2.8. Determination of Inhibition Constants for Non-covalent Inhibitors

The components of competitive and uncompetitive inhibition were determined by measuring the inhibition of enzyme activity in 50 mM sodium phosphate buffer, pH 8.0, over a substrate concentration range of 0.01 – 28 mM and using at least six inhibitor concentrations. Inhibition studies with propidium were carried out in 5 mM sodium phosphate buffer, pH 8.0. Plots of initial velocities versus substrate concentration at a series of inhibitor concentrations were analyzed using Equation 1 to determine the values of $K_m$ and $V_{max}$. Inhibition constants ($K_i$) for each inhibitor were determined by non-linear least squares fitting of plots of $V_{max}/K_m$ versus inhibitor concentration using Equation 2:

$$V_{max}/K_m = \left(\frac{(V_{max}/K_m)K_i}{K_i + [I]}\right)$$  

Equation 2

2.9. Determination of Dissociation Constants for Propidium

Dissociation constants ($K_D$) for the binding of propidium iodide to rHuAChE, HuBChE, and PoBChE were determined by titrating the fluorescence of propidium using a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Titrations were performed in wells of a microtiter plate containing 200 μL of 1.0–2.0 μM enzyme solution in 5 mM Tris.HCl buffer pH 8.0. The fluorescence of the enzyme solution was measured following addition of a small aliquot of propidium, using an excitation wavelength of 520 nm and an emission wavelength of 625 nm. The measured fluorescence was corrected for changes in excitation energy, inner filter effects, and changes in sample volumes during titration by conducting a parallel titration of buffer blank with propidium. The $K_D$ values of propidium for rHuAChE, HuBChE, and PoBChE were calculated using a Scatchard plot. The Scatchard equation is $r/[L] = nK_a - rK_a$ where $L =$ ligand concentration, $n =$ number of ligand sites, $K_a$ is the association constant, and $r = [L_{bound}]/[P]_o$. The symbol $r$ is the ratio of the concentration of bound ligand $[L_{bound}]$ to total available binding sites $[P]_o$. A plot of $r/[L]$ versus $r$ yields the Scatchard plot with a slope of -$K_a$ and a Y-intercept of $nK_a$. The dissociation constant is the inverse of the association constant. To determine if propidium was binding to the active or peripheral anionic site, the active-site of each enzyme was covalently modified by addition of equimolar amounts of paraoxon followed by incubation of the samples at 22 °C for 30 min. These inhibited enzyme samples were used for fluorescence titration as described above.

2.10. pH Dependence of Catalytic Activity

The effect of pH on enzyme activity was determined by measuring activity at various pH values as described. Substrate concentration range from 0.01 to 28 mM was used with the following buffer solutions: 50 mM sodium acetate at pH 5.0 and 5.5; 50 mM sodium phosphate in the pH range 6.0–8.0; and 50 mM glycine in the pH range 8.0–10.0. The kinetic parameters for the hydrolysis of ATC by rHuAChE and BTC by Po and HuBChE were calculated and the pH dependence of $V_{max}$ was fit to a two-$pK_a$ model described by Equation 3:
\[ V_{\text{max}} = \frac{L}{1 + 10^{(pK_1 - pH)} + 10^{(pH-pK_2)}} \]  

Equation 3

\( V_{\text{max}} \) is the apparent velocity at a given pH and \( L \) is \( V_{\text{max}} \) of the reaction. Equation 3 was adapted from \(^{33}\) Segel p. 892 Enzyme Kinetics, Wiley-Interscience 1975.

2.11. Organophosphorylation of Cholinesterases by DFP

rHuAChE, HuBChE and PoBChE were progressively inhibited with four concentrations of DFP. Excess DFP was added to \( 0.50 \times 10^{-7} \) M ChE in 50 mM sodium phosphate buffer, pH 8.0, containing 0.05 % BSA at 25 °C. Aliquots of enzyme-DFP mixtures were withdrawn at various time intervals and assayed for residual activity. The inhibition reaction was pseudo-first order. A plot of the natural log of residual activity versus time gave a straight line. The slope of the line is equal to \( k_{\text{obs}} \). A replot of \( 1/k_{\text{obs}} \) versus \( 1/[\text{DFP}] \) was linear and yielded \( k_i \) from the slope of the line. \(^{34}\) Data were fit to Equation 4 as described. \(^{34}\)

\[ \frac{1}{k_{\text{obs}}} = \frac{K_d}{k_i[\text{DFP}](1-\alpha)} + \frac{1}{k_i} \]  

Equation 4

where \( \alpha \) is defined by Equation 5, and \([S]\) is substrate concentration.

\[ \alpha = \frac{[S]}{K_m + [S]} \]  

Equation 5

2.12. Reactivation of DFP-Inhibited Cholinesterases by 2-PAM

Second-order rate constants (\( k_r \)) for the reactivation of ChE-DFP conjugates by 2-PAM were determined as described \(^{35}\). ChE-DFP conjugates were prepared by incubating enzymes in 0.1 M TAPS buffer, pH 9.0, with a 10-fold molar excess of DFP for 30 min at 22 °C resulting in an inhibition of 95–98% of the starting activity. Excess DFP was removed using Bio-Spin® 6 columns. Aliquots of DFP-inhibited enzyme were diluted with 50 mM sodium phosphate buffer, pH 8.0, containing 0.05% BSA and allowed to reactivate by the addition of 0.01 to 3 mM concentrations of 2-PAM. \(^{35,36}\) Samples were incubated at 25 °C and 10 μL of each mixture was withdrawn at various time intervals and assayed for the recovery of activity. Percent reactivation was determined by comparing reactivated enzyme activity of inhibited sample with uninhibited control sample. A single exponential association equation was used to calculate \( k_{\text{obs}} \) for each oxime concentration using Equation 6. \(^{37}\)

\[ V_t = V_o \left(1 - e^{-k_{\text{obs}}t}\right) \]  

Equation 6

where \( v_t \) is activity at time \( t \), \( v_o \) is the starting enzyme activity, \( k_{\text{obs}} \) is the rate constant of reactivation, and \( t \) is time after addition of oxime. \(^{37}\) A replot of \( k_{\text{obs}} \) versus 2-PAM concentration was used to determine \( k_r \) using Equation 7.
\[ k_{\text{obs}} = \frac{k_r [2 - \text{PAM}]}{K_D + [2 - \text{PAM}]} \]  
Equation 7

where \( k_r \) is the reactivation rate constant and \( K_D \) is the dissociation constant between 2-PAM and the enzyme.\(^{37}\)

### 2.13. Aging of DFP-Inhibited Cholinesterases

Aging rate constants for DFP-inhibited ChEs were measured as described.\(^{38}\) ChE-DFP conjugates were prepared in 0.1 M TAPS buffer pH 9.0 as described above and aliquots of inhibited enzymes were withdrawn at various time intervals and transferred to tubes containing 10 μL of 1 mM 2-PAM solution. Samples were incubated for 24 h at 22 °C and recovered activity was measured with 1 mM ATC.\(^{25}\) The aging rate constants were determined from Equation 8 for first-order decay:

\[ \% (E_{\text{react}})_t = A e^{-k_a \cdot t} \]  
Equation 8

where \( k_a \) is the aging rate constant and \( t \) is the time at which the inhibition reaction was stopped by 2-PAM.

### 3 RESULTS

#### 3.1. Isolation of Butyrylcholinesterase from Porcine Milk

Unlike human plasma (4 U/mL), porcine plasma contains very little BChE activity (0.2 U/mL). On the other hand, porcine milk was found to contain 1–3 U/mL of BChE activity. Therefore, milk was used as the source of PoBChE for this study. PoBChE was purified using high speed centrifugation to remove fat, followed by procainamide affinity and gel permeation chromatography. Approximately 3000 U of purified PoBChE with an overall yield of 39% were recovered from 2000 mL of porcine milk. The specific activity of various preparations ranged from 360 to 650 U/mg.

The majority of the enzyme was tetrameric in form. It eluted in a major peak from a YMC-Pack Diol-300 size exclusion column (0.6 cm x 30 cm) with a retention time of 7.51 min (Figure 1), similar to the retention time of tetrameric HuBChE. The small shoulder at 8.8 min had BChE activity and could be a monomer or dimer of BChE.

SDS gel electrophoresis of reduced and denatured PoBChE purified by Hupresin affinity chromatography showed a major band at 85 kDa for the PoBChE monomer and a weak band at 170 kDa for the nonreducible PoBChE dimer (Figure 2). A similar pattern of bands was observed for pure HuBChE. These results suggest that the molecular weight of tetrameric PoBChE is similar to the 340,000 Da of the HuBChE tetramer. Sedimentation equilibrium ultracentrifugation of BChE purified from porcine parotid gland yielded a molecular weight...
of 342,000 to 353,000 Da, supporting our conclusion that PoBChE in porcine milk is a tetramer of 340,000 Da.

### 3.2. Substrate Specificity

The catalytic parameters of rHuAChE, HuBChE, and PoBChE were determined using three positively-charged substrates, ATC, PTC, and BTC. Activity curves shown in Figure 3 were analyzed by fitting the data to Equation 1 (for rHuAChE and HuBChE) or the Haldane equation (for PoBChE); the catalytic parameters are listed in Table 1. HuBChE showed marked substrate activation with ATC, PTC, and BTC, as indicated by $b$ values of 2.2 to 3.2, while rHuAChE showed substrate inhibition, as indicated by $b < 1$. On the other hand, PoBChE did not show substrate activation, as would be expected for a BChE. Rather a slight inhibition was noted at substrate concentrations >7 mM. In fact, many attempts to fit multiple data sets with PoBChE to Equation 1 failed; therefore catalytic parameters were determined by fitting the data to the Haldane equation. The Haldane equation $v = V_{max} / (1 + Km/[S] + [S]/Ks)$ reduces to $v = V_{max} / (1 + [S]/Ks)$ when $[S] \gg Km$ where $S$ is the substrate concentration. The $K_m$ values for PoBChE were almost 10-fold higher than those for HuBChE, for all three positively-charged substrates tested.

The $k_{cat}$ values for rHuAChE, HuBChE, and PoBChE were determined by normalizing $V_{max}$ to the number of active-sites, determined by direct titration with DEPQ, as described. Consistent with previous reports, $k_{cat}$ values for rHuAChE were 8- to 10-fold higher than those for HuBChE. The $k_{cat}$ value for HuBChE with BTC was 30,000 min$^{-1}$, which is similar to that reported in the literature. Although $k_{cat}$ values for PoBChE were 1.5- to 2-fold higher than those for HuBChE, the higher $K_m$ values for PoBChE resulted in much lower $k_{cat}/K_m$ values for this enzyme with all three charged substrates tested. Consistent with reported observations, the catalytic efficiency of HuBChE measured in terms of $k_{cat}/K_m$ increased with an increase in length of the acyl chain of the substrate (BTC > PTC > ATC). The catalytic efficiency of PoBChE, on the other hand, was not affected by the size of the acyl chain, and all three charged substrates were turned over at similar rates. The catalytic efficiency of rHuAChE decreased with an increase in length of the acyl chain of the substrate.

### 3.3. pH Dependence of Catalytic Activity

The catalytic activities of rHuAChE, HuBChE, and PoBChE as a function of pH are shown in Figure 4. The bell shaped curves show maximum activity between pH 7.5 and 8.5. The curves were fit to a two $pK_a$ mode, Equation 3, yielding calculated $pK_1$ and $pK_2$ values for HuBChE of 6.65 ± 0.06 and 8.93 ± 0.15, respectively. rHuAChE displayed maximum catalytic activity at pH 8 with calculated $pK_1$ and $pK_2$ values of 6.65 ± 0.06 and 9.99 ± 0.06, respectively. For PoBChE, the maximum catalytic activity was observed between pH 8 to 9 and the calculated $pK_1$ and $pK_2$ values were 5.92 ± 0.01 and 9.10 ± 0.06. The $pK$ value of ~6 is attributed to ionization of His 438 in the catalytic triad. A similar broad pH dependence for porcine BChE activity has been reported, with maximum activity in the pH range 7.5 to 9.
3.4. Binding of Inhibitors

To further understand differences between HuBChE and PoBChE, inhibition studies were conducted with various known inhibitors of AChE and BChE. The $K_i$ values summarized in Table 2 are discussed below. The reversible inhibitors exhibit mixed-type behavior, however only the competitive component is reported in Table 2.

3.5. AChE-Specific Inhibitors

The two AChE-specific inhibitors that were examined were (−) huperzine A and edrophonium. As expected for a BChE species, PoBChE displayed a $K_i$ value for (−) huperzine A (28.61 μM) that was much higher than that for rHuAChE, but was similar to the $K_i$ value of 75.6 μM for HuBChE (Table 3). Similarly, inhibition studies with edrophonium, yielded a $K_i$ value of 18.48 μM for PoBChE, which was within 3-fold of that for HuBChE, while being higher than that for rHuAChE. The $K_i$ values of (−) huperzine A and edrophonium for rHuAChE were 20,000-fold and 100-fold lower than those for HuBChE. It was concluded that the enzyme purified from porcine milk is resistant to AChE-specific inhibitors and is therefore classified as BChE.

3.6. BChE-Specific Inhibitors

Three BChE-specific inhibitors, ethopropazine, iso-OMPA, and tacrine were examined. Ethopropazine and tacrine display the most selectivity and the least selectivity for HuBChE, respectively. Inhibition studies with ethopropazine, a substituted phenothiazine, revealed a 1,280-fold difference in $K_i$ between rHuAChE and HuBChE (Table 2). The $K_i$ of ethopropazine for PoBChE was within 4-fold that for HuBChE. An approximately 80 fold higher $K_i$ value was observed for the inhibition of rHuAChE by iso-OMPA compared to HuBChE and PoBChE. Since iso-OMPA is an irreversible inhibitor, the values in Table 2 are apparent $K_i$.

The $K_i$ value for inhibition of PoBChE by tacrine was approximately an order of magnitude lower (0.43±0.07 nM) than for HuBChE (8±2 nm). It was concluded that the enzyme in porcine milk was inhibited by BChE-specific inhibitors and is therefore classified as BChE.

3.7. Bisquaternary Inhibitors

Three bisquaternary inhibitors, propidium, decamethonium, and BW284c51 were examined for binding to rHuAChE, HuBChE and PoBChE. Propidium, a bisquaternary ligand that primarily interacts at the peripheral anionic site of AChE, has been reported to bind to the active-site rather than to the peripheral anionic site of BChE. Consistent with previous observations, inhibition studies with propidium in 5 mM sodium phosphate, pH 8.0, showed that propidium was an uncompetitive inhibitor of rHuAChE and a competitive inhibitor of HuBChE and PoBChE (data not shown). However, propidium displayed a 29-fold lower affinity for PoBChE compared to HuBChE. To further identify the site of interaction of propidium, fluorescence titration studies were conducted with rHuAChE, HuBChE, and PoBChE, and $K_D$ values were calculated. Consistent with the observed $K_i$ values, the $K_D$ values of propidium for rHuAChE, HuBChE, and PoBChE were determined to be 0.47 ± 0.1, 0.31 ± 0.02, and 1.50 ± 0.23, respectively. As shown in Figure 5, diethoxyphosphorylation of the active site serine with paraoxon significantly reduced the
binding of propidium to HuBChE and PoBChE (panels B and C), but had less effect on rHuAChE (panel A). These results confirm previous observations that propidium primarily interacts at the peripheral anionic-site of AChE, but at the active-site of BChE.

Unlike propidium, where the quaternary nitrogens are maximally separated by a distance of 4.8 Å, the quaternary nitrogens in decamethonium and BW284c51 are maximally separated by 14 Å. Consistent with published observations, the $K_i$ of decamethonium for rHuAChE and HuBChE were similar (Table 2). However, decamethonium was 100-fold less potent for PoBChE than for HuBChE. For BW284c51, an AChE-specific inhibitor, a 655-fold difference in $K_i$ was observed between rHuAChE and HuBChE (Table 2). The $K_i$ for PoBChE was within 5-fold of that for HuBChE. It was concluded that the peripheral anionic site in PoBChE has properties similar to the peripheral anionic site in HuBChE.

### 3.8. Inhibition, Aging, and Reactivation Kinetics with DFP

The bimolecular rate constants for the inhibition of rHuAChE, HuBChE, and PoBChE by DFP and rate constants for aging as well as reactivation of DFP-inhibited enzymes by 2-PAM are shown in Table 3. The inhibition rate constant ($k_i$) for PoBChE of $5.3 \times 10^5$ M$^{-1}$ min$^{-1}$ was similar to that for rHuAChE, and approximately 50-fold lower than that for HuBChE. This means that HuBChE is more sensitive to inhibition by DFP than rHuAChE, a result consistent with the literature. In contrast, PoBChE is 50-fold less sensitive to inhibition by DFP than HuBChE. The aging rate constants $k_a$ of DFP-inhibited PoBChE $137 \times 10^{-5}$ min$^{-1}$ and rHuAChE $129 \times 10^{-5}$ min$^{-1}$ were similar, but 5-fold lower than for HuBChE. The second order rate constants $k_r$ for the reactivation of DFP-inhibited HuBChE and PoBChE by 2-PAM, were 10- and 50-fold higher than that for rHuAChE.

### 3.9 Porcine BChE Amino Acid Sequence

Figure 6 shows the amino acid sequence of milk-derived PoBChE taken from the deleted NCBInr 2015.3.10 database, entry XP_003358712 and 335299867. The 28 amino acid signal peptide has been removed because it is absent in the mature, secreted protein. Mass spectrometry (LC-MS/MS) confirmed the identity of the sequence in Figure 6.

PoBChE and HuBChE each contain 574 amino acids. Their protein sequences are 91% identical, supporting the conclusion that the esterase in porcine milk is PoBChE. Residues known to be important for the function and structure of HuBChE are conserved.

Residues boxed in Figure 6 include the catalytic triad (S198, E325, H438), the cation-π binding site (W82), the peripheral anionic binding site (D70, Y332), the acyl binding pocket (L286, V288, W231), and salt bridges (R42-E90, R147-D170, R265-E161, R515-D395). Important residues that are not boxed include the oxyanion hole (G116, G117, A199), three intra-chain disulfide bonds (C65-C92, C252-C263, C400-C519), and one interchain disulfide bond (C571-C571). Glycans are attached to 8 asparagines in PoBChE (N57, N106, N241, N256, N341, N455, N481, N486), but to 9 asparagines in HuBChE (N17, N57, N106, N241, N256, N341, N455, N481, N486).

Computational analysis identified 47 residues that are important for BChE function and structure. Four residues in this set are different in PoBChE (V77, V277, L285, I398) compared to HuBChE (H77, A277, P285, F398). H77 makes a hydrogen bond with M81 in
the Omega loop. A277 is in the peripheral anionic site at the mouth of the active site gorge. Residues 285 and 398 line the active site gorge.

A patch of 4 residues at 282–285 near the acyl-binding pocket is different in the two enzymes. One positively-charged end of decamethonium interacts with W82 and D197, while the main chain faces the acyl-binding pocket of HuBChE. The sequence differences in the 282–285 patch may be responsible for the 100-fold higher $K_i$ value of decamethonium for PoBChE compared to that for HuBChE (Table 2).

3.10. Binding of PoBChE to Anti-HuBChE Antibodies

Four anti-HuBChE monoclonal antibodies were tested for ability to bind PoBChE. It was found that monoclonal antibodies 11D8, mAb2 and 3E8, but not B2 18–5, bound PoBChE. Figure 7 shows ELISA data for monoclonal antibody 11D8. The lowest PoBChE concentration tested, 0.0625 ng in 100 μL (0.0004375 units/mL), had an absorbance at 405 nm of 0.122 after 60 min reaction with BTC/DTNB, which is higher than the 0.096 value for control wells with no PoBChE. PoBChE activity developed fastest with immobilized mAb2 (5-fold faster than with 3E8), slower with 11D8 (2.5-fold faster than with 3E8), and slowest with 3E8, suggesting that 1 μg mAb2 bound 2 times more PoBChE than was bound by 1 μg 11D8, and 5-times more PoBChE than was bound by 1 μg 3E8.

3.11. Polyproline-Rich Peptides in the PoBChE Tetramer

All BChE and AChE tetramers studied to date are composed of 4 identical subunits assembled through non-covalent interaction with a polyproline-rich peptide. The C-terminal 40 residues of AChE and BChE constitute the tetramerization domain that is required for binding the polyproline-rich peptide. Amino acid analysis of PoBChE purified from porcine parotid gland showed an unusually high proline content of 7.6%, suggesting that PoBChE tetramers include a tetramer organizing polyproline-rich peptide. Mass spectrometry analysis of tetrameric PoBChE identified the polyproline-rich peptides listed in Table 4. The polyproline-rich peptides derived from 12 proteins. Two proteins, Zinc finger homeobox protein 4 and Proline-rich Protein 12, each contributed 2 different polyproline-rich peptides. The most abundant polyproline-rich peptide originated from acrosin where the peptide count was 138 and the least abundant from proline-rich protein 16 where the peptide count was 1. The polyproline-rich peptides listed in Table 4 are a composite of the peptides identified by mass spectrometry. For example, the 27 residue peptide in acrosin was deduced from the 25 overlapping peptides listed in Supporting Information Table S1, ranging in length from 11–19 residues. Supporting Information Tables S1-S12 list all the polyproline-rich peptides identified in PoBChE tetramers.

4 DISCUSSION

4.1. Substrate specificity

PoBChE was isolated from porcine milk and its catalytic and inhibitory properties were compared with rHuAChE and HuBChE. The best substrate for rHuAChE was ATC, and for HuBChE was BTC. However, PoBChE hydrolyzed ATC, BTC, and PTC with similar $K_m$ and $k_{cat}$ values. Steady state kinetic studies revealed that the $k_{cat}/K_m$ values for PoBChE are...
10-fold lower than for \( \text{HuBChE} \). This suggests the active site pocket in \( \text{PoBChE} \) is less efficient at orienting positively charged compounds for hydrolysis compared to \( \text{HuBChE} \). The difference in amino acid sequence at positions 282–285 could have a role, since residues 282–285 are part of the lining of the active site gorge and therefore interact directly with substrates. Our substrate specificity studies identified the esterase in porcine milk as \( \text{ChE} \), but did not clearly identify it as \( \text{BChE} \).

\( \text{HuBChE} \) displayed substrate activation with all three substrates, whereas \( \text{PoBChE} \) displayed substrate inhibition at concentrations greater than 7 mM. This result was surprising as it is known that \( \text{HuBChE} \) shows substrate activation with BTC up to 20 mM, whereas \( \text{AChE} \) shows substrate inhibition at concentrations of ATC greater than 1 mM. Both phenomena are thought to be due to the binding of a substrate molecule at a peripheral anionic site remote from the catalytic site. The binding of substrate at the peripheral anionic site allosterically affects conformation at the active site thus influencing acylation and/or deacylation rates. Using site-directed mutagenesis studies, the residues involved in substrate activation of \( \text{HuBChE} \) were identified as D70, W82, E197, Y332, and A328. All these residues are conserved in \( \text{PoBChE} \). It was concluded that the substrate activation results did not prove the esterase in porcine milk is \( \text{PoBChE} \).

### 4.2. Inhibitor specificity

Inhibition studies with \( \text{BChE} \)-specific inhibitors showed that \( \text{PoBChE} \) was inhibited by ethopropazine and iso-OMPA with \( K_i \) values similar to those for \( \text{HuBChE} \). However, \( \text{AChE} \)-specific inhibitors (−) huperzine A, edrophonium, and BW284c51 were poor inhibitors. This result supported the conclusion that the esterase isolated from porcine milk is \( \text{PoBChE} \) and is not \( \text{PoAChE} \).

\( \text{PoBChE} \) was more sensitive to inhibition by tacrine compared to \( \text{HuBChE} \). \( \text{PoBChE} \) was less sensitive to propidium and decamethonium compared to \( \text{HuBChE} \). The increased affinity of tacrine to \( \text{PoBChE} \) could be due to differences in amino acid residues in the active-site, in particular at positions 282–285. Previous site-directed mutagenesis studies with \( \text{AChE} \) mutants showed that the cluster of aromatic amino acid residues, Y72(70), Y124(121), and W286(279), located at the lip of the gorge stabilizes the binding of substrates and inhibitors like BW284C51, decamethonium and propidium. Note that the italic numbers in brackets refer to the positions of corresponding residues in the \( \text{BChE} \) sequence. Although W286(279) is replaced by Ala in \( \text{HuBChE} \), consistent with previous observations, there was no difference in the inhibition of \( \text{HuAChE} \) and \( \text{HuBChE} \) by decamethonium. The orientation of decamethonium in the larger \( \text{BChE} \) active site gorge is different from that in the \( \text{AChE} \) gorge. Since these residues at the peripheral anionic site are conserved in \( \text{PoBChE} \), the reduced affinity of decamethonium for \( \text{PoBChE} \) is likely due to differences in the active-site. The relatively larger active-site gorge of \( \text{HuBChE} \) can accommodate propidium, making it a competitive inhibitor of \( \text{BChE} \). Therefore, the reduced affinity of propidium for \( \text{PoBChE} \) is also due to differences in the active-site.

\( \text{PoBChE} \) and \( \text{rHuAChE} \) were relatively insensitive to inhibition by the OP toxicant DFP compared to \( \text{HuBChE} \), their rate constants for inhibition being approximately 40- and 150-fold slower than the second order rate constant of \( 229 \times 10^5 \text{ M}^{-1} \text{ min}^{-1} \) for \( \text{HuBChE} \). DFP is
considered to be a BChE-specific inhibitor since BChE in human plasma is very much more sensitive to inhibition than AChE in human brain and erythrocytes.63, 64 This difference can be seen in other examples. The DFP concentration that inhibits 50% of the horse erythrocyte (AChE) activity in 30 min is 270-fold higher than that for horse plasma enzyme (BChE).45 The inhibitory power, $k_i$, of DFP for HuBChE is 150 times greater than for bovine erythrocyte AChE.44

The rate constant for aging of DFP-inhibited enzyme was similar for PoBChE and rHuAChE, but 7-fold faster for HuBChE. However, the reactivation of DFP-inhibited enzyme by 2-PAM was similar for PoBChE and HuBChE. Overall, our studies with DFP did not provide conclusive proof that the esterase in porcine milk is PoBChE.

### 4.3. Amino acid sequence and antibody reactivity

Conclusive evidence that the esterase isolated from porcine milk is PoBChE was provided by amino acid sequencing of the purified enzyme. The sequence was 91% identical to that of HuBChE (P06276), 100% identical to that of Sus scrofa BChE (gi335299867 in the deleted NCBI databases for the years 2013 and 2015), 100% identical to NP_001344438.1 in the November 2017 NCBI database, and only 52% identical to that of PoAChE (gi335284162) in the deleted NCBIr 2013.6.17 and 2015.3.10 databases. Additional support was provided by the observation that anti-HuBChE monoclonal antibodies recognized PoBChE. This result is in agreement with Augustinsson and Olsson that porcine milk contains only one esterase and that the esterase is BChE.20

### 4.4. Polyproline-rich peptides in PoBChE

Soluble tetramers of human and equine BChE, and of fetal bovine AChE consist of 4 identical subunits plus one polyproline-rich peptide per tetramer.51, 52, 54–56 Membrane-anchored BChE and AChE tetramers are assembled into tetramers through noncovalent interaction with a polyproline-rich region of the proteins COLQ or PRIMA.65, 66 Whereas the membrane-anchored tetramers contain a specific embedded polyproline-rich protein, the soluble tetramers contain peptides from a variety of polyproline-rich proteins. The short polyproline-rich peptides in the soluble tetramers are fragments derived from degradation of proteins that are normally present in the cytoplasm or nuclei of cells. It has been shown that soluble BChE and AChE tetramers can scavenge whatever polyproline-rich peptide is available and incorporate that peptide into their structure.26, 67–70 This view of the identity and origin of polyproline-rich peptides in BChE tetramers is supported by our results for PoBChE tetramers.

PoBChE tetramers contained 14 different polyproline-rich peptides that originated from 12 different proteins. The set of polyproline-rich peptides in PoBChE purified from porcine milk was different from the set of polyproline-rich peptides in HuBChE55, 56 and equine BChE purified from plasma.52 It was also different from the set of polyproline-rich peptides in recombinant HuBChE tetramers expressed in Chinese Hamster Ovary cells.26 We interpret this to mean that the amino acid sequence of the polyproline-rich peptides present in soluble tetramers depends on the tissue that synthesizes the BChE or AChE. Porcine milk proteins are synthesized in the mammary glands in breast, whereas plasma proteins are
synthesized in the liver. This leads to the expectation that the protein donors of the polyproline-rich peptides in milk-derived PoBChE are synthesized in breast tissue. The GTEx portal website (https://www.gtexportal.org/home/gene) shows that every protein in Table 4 is expressed in human breast tissue and by implication in porcine breast.

BChE is known as a bioscavenger of nerve agents and organophosphorus pesticides. Our results are consistent with a second bioscavenger role of BChE in scavenging polyproline-rich peptides released from nuclear and cytoplasmic proteins during cell degradation. Whereas scavenging of toxicants results in loss of BChE activity, scavenging of polyproline-rich peptides stabilizes BChE by assembling the subunits into tetramers.

5 Conclusions

Porcine milk contains BChE and is a richer source of BChE than porcine plasma. The biochemical properties of PoBChE are similar to those of HuBChE, but not identical. The major similarities are 1) PoBChE can be purified by affinity chromatography on procainamide and Hupresin Sepharose, 2) PoBChE is inhibited by BChE-specific inhibitors, 3) PoBChE hydrolyzes ATC, BTC, and PTC, 4) the peripheral anionic site in PoBChE has properties similar to the peripheral anionic site in HuBChE, 5) the amino acid sequence of PoBChE is 91% identical to that of HuBChE, 6) 3 anti-HuBChE monoclonal antibodies recognize PoBChE, 7) PoBChE is a tetramer of 4 identical subunits assembled through noncovalent interaction with a polyproline-rich peptide, 8) the molecular weight of the PoBChE tetramer is 340 kDa.

PoBChE differs from HuBChE as follows: 1) PoBChE does not prefer BTC over ATC and PTC, 2) there is not a clear substrate activation phase for PoBChE, 3) unlike HuBChE, PoBChE is not supersensitive to inhibition by low concentrations of DFP, 4) PoBChE is resistant to inhibition by decamethonium, but supersensitive to inhibition by tacrine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AChE
acetylcholinesterase

ATC
acetylthiocholine iodide CAS 1866–15-5
**BChE**
butyrylcholinesterase

**BSA**
bovine serum albumin

**BTC**
butyrylthiocholine iodide CAS 1866–16-6

**BW284c51**
1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide CAS 402–40-4

**ChE**
cholinesterase

decamethonium bromide
decane-1,10-bis(trimethylammonium bromide) CAS 541–22-0

**DEPQ**
7-(O,O-diethyl-phosphinyloxy)-1-methylquinolinium methylsulfate

**DFP**
diisopropyl fluorophosphates CAS 55–91-4

**DTNB**
5, 5’-dithiobis (2-nitrobenzoic acid) CAS 69–78-3

**edrophonium chloride**
ethyl(m-hydroxyphenyl)dimethylammonium chloride CAS 116–38-1

**ELISA**
enzyme-linked immunosorbent assay

**ethopropazine hydrochloride**
10-[2-diethylaminopropyl]phenothiazine hydrochloride CAS 1094–08-2

**rHuAChE**
recombinant human acetylcholinesterase P22303

**HuBChE**
Human butyrylcholinesterase P06276

**iso-OMPA**
tetra(monoisopropyl)pyrophosphortetramide CAS 513–00-8

**LC-MS/MS**
liquid chromatography-tandem mass spectrometry

**OP**
organophosphorus toxicant
2-PAM
pyridine-2-aldoxime methiodide CAS 94–63-3

paraoxon ethyl
(O,O-diethyl O-(4-nitrophenyl) phosphate) CAS 311–45-5

PBS
phosphate buffered saline

PNGaseF
peptide-N-glycosidase F

PoBChE
porcine milk butyrylcholinesterase

propidium iodide
3,8-diamino-5’–3’-(trimethylammonium)propyl-6-phenylphenanthridinium iodide CAS 25535–16-4

PTC
propionylthiocholine iodide CAS 1866–73-5

tacrine
9-amino-1,2,3,4-tetrahydroacridine hydrochloride CAS 1684–40-8

TAPS
N-[tris(hydroxymethyl)methyl]-3-amino propanesulfonic acid CAS 29915–38-6

TBS
tris-buffered saline, 20 mM Tris.HCl pH 7.4 with 0.15 M NaCl

TBST
tris-buffer saline plus 0.05% Tween-20

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Highlights

- Porcine milk is a richer source of BChE than porcine plasma.
- PoBChE is a tetramer assembled through a polyproline-rich peptide.
- PoBChE is purified on procainamide Sepharose or Hupresin affinity gel.
- PoBChE amino acid sequence is 91% identical to HuBChE.
- PoBChE does not prefer butyryl-over acetyl- and propionylthiocholine.
Figure 1.
Elution profile of PoBChE from a size exclusion YMC-Pack Diol-300 column. Pure PoBChE eluted at 7.5 min, the same time as pure tetrameric HuBChE, indicating that the peak at 7.5 min represents tetrameric PoBChE. The shoulder at 8.8 min had BChE activity.
Figure 2. SDS gel electrophoresis of Hupresin-purified PoBChE. Contaminating proteins, including some PoBChE, were washed off with 0.3 M NaCl (lane 1). Pure PoBChE was eluted with 0.1 M tetramethylammonium bromide (lane 2) followed by 2 M NaCl (lane 3). Pure HuBChE (lane 5) monomers at 85 kDa and dimers at 170 kDa have the same size as PoBChE.
Figure 3.
Substrate concentration dependence of activity for rHuAChE, HuBChE and PoBChE: ▲ ATC, ■ PTC, □ BTC.
Figure 4.
pH dependence of the catalytic activity of rHuAChE, HuBChE and PoBChE. Enzyme activities were determined in various buffers at 25 °C and expressed as a percent of highest activity for each enzyme: ○ rHuAChE; ▲ HuBChE; ■ PoBChE.
Figure 5.
Titration of rHuAChE, HuBChE and PoBChE with propidium using un-modified enzymes (■) as well as with paraoxon-inhibited enzymes (▲).
Figure 6.
The amino acid sequence of the protein purified from porcine milk is 91% identical to the amino acid sequence of HuBChE. Mature BChE proteins without the 28 amino acid signal peptide are shown (UniProt accession P06276 for HuBChE and NCBI accession XP_003358712 and 335299867 for PoBChE). Residues were identified by LC-MS/MS of trypsin digested PoBChE. The amino acid sequence of PoBChE has been deposited in the NCBI database under accession number NP_001344438.1. The function of boxed residues is explained in the text.
Figure 7.
Binding of PoBChE to immobilized anti-HuBChE monoclonal antibody 11D8 in a 96-well microtiter plate. Antibody-bound PoBChE hydrolyzed butyrylthiocholine, yielding the yellow color detected at 405 nm.
Table 1.

Steady State Kinetic Constants for rHuAChE, HuBChE and PoBChE

| ChE      | Subst | $K_{m}$, mM | $K_{so}$, mM | $b$     | $k_{cat}$ min$^{-1}$ | $k_{cat}/K_{m}$ min$^{-1}$ mM$^{-1}$ | Units/ nmole |
|----------|-------|-------------|--------------|---------|----------------------|--------------------------------------|-------------|
| rHuAChE$^a$ | ATC   | 0.11±0.01   | 6.4±1.1      | 0.10±0.01| 431±35 x10$^3$       | 3888±388 x10$^3$                      | 360         |
| rHuAChE$^a$ | PTC   | 0.15±0.01   | 4.7±1.6      | 0.11±0.03| 223±20 x10$^3$       | 1512±126 x10$^3$                      |             |
| rHuAChE$^a$ | BTC   | ND          | ND           | ND      | ND                   | ND                                   | ND          |
| HuBChE$^a$ | ATC   | 0.031±0.004 | 1.7±0.3      | 2.23±0.12| 23±2 x10$^3$         | 697±14 x10$^3$                       |             |
| HuBChE$^a$ | PTC   | 0.017±0.006 | 1.5±0.4      | 3.26±0.27| 32±2 x10$^3$         | 1510±42 x10$^3$                      |             |
| HuBChE$^a$ | BTC   | 0.015±0.003 | 0.7±0.1      | 3.20±0.60| 30±3 x10$^3$         | 1943±178 x10$^3$                     | 60          |
| PoBChE$^b$ | ATC   | 0.37±0.06   | 336±167      | ND      | ND                   | 155±9 x10$^3$                        |             |
| PoBChE$^b$ | PTC   | 0.25±0.01   | ND           | ND      | 46±1 x10$^3$         | 185±11 x10$^3$                       |             |
| PoBChE$^b$ | BTC   | 0.23±0.04   | 287±35       | ND      | 43±1 x10$^3$         | 172±6 x10$^3$                        | 35          |

$^a$Catalytic parameters were calculated using equation 1.

$^b$Catalytic parameters were calculated using the Haldane equation, therefore $b$ values could not be determined. $k_{cat}$ was calculated as $V_{max}/[Enzyme]$.

ND: not determined.
Table 2.

Inhibition constants for rHuAChE, HuBChE and PoBChE

| Inhibitor          | rHuAChE, $K_i$ (μM) | HuBChE, $K_i$ (μM) | PoBChE, $K_i$ (μM) |
|--------------------|---------------------|--------------------|--------------------|
| Ethopropazine      | 65.3±33.1           | 0.05±0.005         | 0.19±0.01          |
| Iso-OMPA           | 4.6±0.6             | 0.05±0.01          | 0.06±0.02          |
| Tacrine            | 0.037±0.002         | 0.008±0.002        | 0.00043±0.00007    |
| (−) Huperzine A    | 0.00032±0.00006     | 75.6               | 28.6±2.2           |
| Edrophonium        | 0.36±0.01           | 50.4±11.7          | 18.5±2.2           |
| BW284c51           | 0.003±0.0001        | 1.9±0.3            | 9.3±0.4            |
| Propidium          | 0.49±0.10           | 0.40±0.03          | 11.7±3.6           |
| Decamethonium      | 2.2±0.1             | 0.8±0.1            | 87.5±15.4          |

Assays were performed in duplicate.
Table 3.
Inhibition, Aging, and Reactivation rate Constants with DFP

| Enzyme   | Inhibition $k_i$, M$^{-1}$ min$^{-1}$ | Aging $k_{ai}$, min$^{-1}$, ($t_{1/2}$) | Reactivation by 2-PAM $k_r$, M$^{-1}$ min$^{-1}$ |
|----------|--------------------------------------|------------------------------------------|--------------------------------------------------|
| rHuAChE  | 1.33±0.11 × 10$^5$                   | 129 × 10$^{-5}$, (t$^{1/2}$ 539 min)     | 11.0                                              |
| HuBChE   | 229±10 × 10$^3$                     | 820 × 10$^{-5}$, (t$^{1/2}$ 85 min)      | 541 ± 23                                          |
| PoBChE   | 5.27±1.03 × 10$^5$                  | 137 × 10$^{-5}$, (t$^{1/2}$ 506 min)     | 108 ± 4                                           |

Assays were performed in triplicate.
Table 4.

Polyproline-rich peptides in PoBChE tetramers

| Name                                                         | Gene      | Peptide                                      | Length | MW     | Peptide count |
|--------------------------------------------------------------|-----------|----------------------------------------------|--------|--------|---------------|
| Acrosin                                                      | ACR       | PAPPPAPPPPPPPPPPPPPPPPPPPPPQQ               | 27     | 2648.4 | 138           |
| Homeobox protein HoxB4                                      | HOXB4     | RDPPPPPPPPPPPPPPPPPPPPPPGL                  | 21     | 2069.1 | 116           |
| Lysine-specific demethylase 6B                              | KDM6B     | PLPPPLPPPPPPPPPPPPPPPPPPPPPLGLAT            | 28     | 2737.5 | 210           |
| Zinc finger homeobox protein 4                              | ZFHX4     | TPPPPPPPPPPPPPPPPPPPPPPPPSA                 | 22     | 2121.1 | 71            |
| Zinc finger homeobox protein 4                              | ZFHX4     | TPPPPPPPPPPPPPPPPPPPPPPSSL                  | 18     | 1764.9 | 71            |
| Zinc finger CCCH type containing protein 4                  | ZC3H4     | GPPPPPPPPPPPPPPPPPPPPPPQM                   | 19     | 1806.9 | 33            |
| Disabled homolog 2-interacting protein-like isoform 1       | DAB2IP    | IDQPPPPPPPPPPPPAPPR                         | 16     | 1668.9 | 12            |
| Protein FAM171A2                                            | FAM171A2  | AAAPPPPPPPPPPPPPPR                          | 17     | 1622.9 | 4             |
| FH2 domain-containing protein 1                             | FHDC1     | PPHSSPPPPPPPPPP                           | 14     | 1366.7 | 10            |
| Proline-rich protein 12                                      | PRR12     | APPPPPPPPPPPPPPASEPK                       | 19     | 1863.0 | 123           |
| Proline-rich protein 12                                      | PRR12     | LPPPPPPPPPPPPPPPPPPPPPPPPPPPP              | 20     | 1975.1 | 123           |
| WAS/WASL-interacting protein family member 2 isoform X1     | WIPF2     | MIIPPPPPPPPPPPPPPPPTF                      | 19     | 1926.0 | 6             |
| Proline-rich protein 16                                      | PRR16     | PNPPPPPPPR                                 | 9      | 967.5  | 1             |
| Proline-rich membrane anchor 1, partial                      | PRIMA1    | PPPPLPPPPPPPPPPPPPPPR                      | 16     | 1645.9 | 107           |

Peptide count is the total number of polyproline-rich sequences in the LC-MS/MS data set. This number includes peptides that appeared in the data more than once. For example, 25 peptides associated with acrosin appeared 138 times, see Supporting Information Table S1.

MW is the molecular weight of the peptide.