Characterization of butyrylcholinesterase in bovine serum

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Abstract

Human butyrylcholinesterase (HuBChE) protects from nerve agent toxicity. Our goal was to determine whether bovine serum could be used as a source of BChE. Bovine BChE (BoBChE) was immunopurified from 100 mL fetal bovine serum (FBS) or 380 mL adult bovine serum by binding to immobilized monoclonal mAb2. Bound proteins were digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry. The results proved that FBS and adult bovine serum contain BoBChE. The concentration of BoBChE was estimated to be 0.04 μg/mL in FBS, and 0.03 μg/mL in adult bovine serum, values lower than the 4 μg/mL BChE in human serum. Nondenaturing gel electrophoresis showed that monoclonal mAb2 bound BoBChE but not bovine acetylcholinesterase (BoAChE) and confirmed that FBS contains BoBChE and BoAChE. Recombinant bovine BChE (rBoBChE) expressed in serum-free culture medium spontaneously reactivated from inhibition by chlorpyrifos oxon at a rate of 0.0023 min⁻¹ (t₁/₂=301 min⁻¹) and aged at a rate of 0.0138 min⁻¹ (t₁/₂=50 min⁻¹). Both BoBChE and HuBChE have 574 amino acids per subunit and 90% sequence identity. However, the apparent size of serum BoBChE and rBoBChE tetramers was much greater than the 340,000 Da of HuBChE tetramers. Whereas HuBChE tetramers include short polyproline rich peptides derived from lamellipodin, no polyproline peptides have been identified in BoBChE. We hypothesize that BoBChE tetramers use a large polyproline-rich protein to organize subunits into a tetramer and that the low concentration of BoBChE in serum is explained by limited quantities of an unidentified polyproline-rich protein.

Graphical Abstract

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1. Introduction

The plasma of humans, monkey, and horse contains BChE (Ralston et al., 1983; Grunwald et al., 1997; Rosenberg et al., 2002). In contrast, the plasma of ruminants (bovine, goat, sheep, reindeer) contains very low levels of an esterase that hydrolyzes butyrylcholine (Augustinsson, 1959). Esterase activity in bovine plasma is almost undetectable when assayed with butyrylcholine, butyrylthiocholine, or propionylthiocholine (Mendel et al., 1943; Augustinsson, 1959; Tecles and Ceron, 2001). Bovine plasma esterase does not hydrolyze benzoylcholine (Mendel et al., 1943; Augustinsson, 1959; Arpagaus et al., 1991), though benzoylcholine is a good substrate for HuBChE. Bovine plasma esterase hydrolyzes $[^3]$Hacetylethanolamine iodide at a rate of 76 nmoles per mL per min at pH 7.4, 30°C (Karanth and Pope, 2003) and acetyltiocholine iodide at a rate of 149 nmoles per mL per min at pH 8, 25°C (Pardio et al., 2001). Based on these observations it is generally accepted that bovine plasma contains acetylcholinesterase (AChE), but not BChE (Mendel et al., 1943; Augustinsson, 1959; Li et al., 2000). BoAChE has been purified from fetal bovine serum (FBS), thus confirming the presence of AChE in FBS (De la Hoz et al., 1986).

Our goal was to re-examine the question of whether bovine plasma contains BoBChE. We provide mass spectrometry evidence for the presence of BoBChE in bovine plasma, though the levels of BoBChE are very low. FBS is a richer source of BoBChE than serum of the adult cow. To address the question of why BoBChE levels in bovine plasma are low, we expressed full-length recombinant bovine BChE (rBoBChE) and studied its characteristics. While titrating rBoBChE with chlorpyrifos oxon, it was noticed that part of the chlorpyrifos oxon-inhibited rBoBChE activity spontaneously recovered. Molecular dynamics simulations provided a mechanism to explain why BoBChE, covalently modified on its active site serine with diepoxide, has the ability to spontaneously regain some activity.

HuBChE is currently the gold standard for studies that aim to protect from the toxicity of nerve agents (Saxena et al., 2006; Mumford et al., 2010; Nachon et al., 2013). The characteristics that make HuBChE the gold standard include the following: 1) HuBChE is not immunogenic to humans, 2) pure HuBChE is stable for years in solution at 4°C, 3) the half-life of HuBChE in the human circulation is about two weeks, and 4) HuBChE
scavenges all types of nerve agents. Purified HuBChE has limited availability from commercial sources, making equine BChE a reasonable alternative. The present work tested the possibility that bovine serum could be another source of BChE. We conclude that bovine serum is not a potential source of plasma BChE because the levels of BoBChE are extremely low.

2. Materials and Methods

2.1. Materials

CNBr-activated Sepharose 4 Fast Flow (Amersham Bioscience, Piscataway, NJ; 17-0981-01). Amicon Ultra-15 centrifugal filter, regenerated cellulose 10,000 NMWL (Merck Millipore UFC901024). Adult bovine serum (Sigma, St. Louis, MO; B9433 and Greater Omaha Packing Co). Fetal bovine serum (FBS) (ThermoFisher Scientific_Gibco; 10437028) AChE purified from FBS was a gift from Dr. Ashima Saxena, Walter Reed Army Institute of Research. Ultraculture (BioWhittaker, Lonza, Walkersville, MD; 12-725F). Chinese Hamster Ovary cells (CHO) (ATCC, Manassas, VA; CCL-61). G418 (geneticin) (ThermoFisher Scientific, Waltham, MA; 11811023). Q Sepharose Fast Flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden; 17-0510-04). Ethopropazine (Sigma, St. Louis, MO; E-2880). Chlorpyrifos oxon (CPO) O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphate, 98.1% pure (ChemService Inc., West Chester, PA; MET-674B). 5,5’-Dithio-bis-(2-nitrobenzoic acid) (DTNB) (Sigma, St. Louis, MO; D8130). S-Butyrylthiocholine iodide (BTC) (Sigma Aldrich_Fluka, St. Louis, MO; 20820). Acetylthiocholine iodide (ATC) (Sigma, St. Louis, MO; A5751). Trypsin sequencing grade modified porcine trypsin (Promega, Madison, WI; V5113). Immulon 2HB 96-well plates (Thermo 3455). Pure HuBChE was purified from outdated human plasma (Lockridge et al., 2005).

2.2. Plasmids for recombinant BoBChE and expression in CHO cells

Four BoBChE plasmids in mammalian expression vector pcDNA3 were constructed by Syd Labs, Inc. (Natick, MA). Plasmids encoded full-length BoBChE (gi:116004026, accession # P32749) but differed in the signal peptide sequence. 1) In the 1st plasmid, nucleotides 64-1929 used the native signal peptide encoding 47 amino acids. 2) In the 2nd plasmid, nucleotides 121-1929 used the native signal peptide encoding 28 amino acids. 3) The 3rd plasmid encoded 47 amino acids in the signal peptide, but the native ATG start site was replaced with the Kozak sequence. 4) The 4th plasmid encoded 28 amino acids in the signal peptide, but the native ATG start site was replaced with the Kozak sequence.

The plasmids were transfected into CHO cells and stable colonies were selected for resistance to G418. Cells were grown in serum-free Ultraculture supplemented with 2 mM L-glutamine and 0.8 mg/mL G418. Standard culture medium containing 10% (v/v) FBS was not used because BChE and AChE in FBS could interfere with interpretation of activity from rBoBChE.

2.3. Antibodies

Mouse anti-human BChE monoclonal mAb2, created in the laboratory of Jacques Grassi (Checler et al., 1990), was purified from ascites fluid using Protein G agarose (ProteinMods,
Madison, WI). Nucleotide and amino acid sequences of the heavy and light chains from mAb2 are deposited in the NCBI database under accession numbers KJ141199 and KJ141200 (Peng et al., 2015).

Syd Labs Inc. expressed the light and heavy chains of mAb2 in HEK293 cells in culture medium containing 10% (v/v) FBS and purified mAb2 on Protein A agarose. Mouse anti-human BChE monoclonal B2 18-5, created in the laboratory of Stephen Brimijoin (Brimijoin et al., 1983), accession numbers KT189143 and KT189144, was purified from serum free culture medium (Peng et al., 2015).

2.4. Crosslinking monoclonals to Sepharose beads

The buffer for monoclonals mAb2 and B2 18-5 was changed to 0.15 M sodium bicarbonate pH 8, 0.5 M NaCl in an Amicon centrifugal filter 10,000 MW cutoff, and the proteins concentrated to 5 mg/mL. CNBr-activated Sepharose (1 g powder) swelled to 3 mL after being washed in ice-cold 1 mM HCl to remove preservatives and then washed in coupling buffer (ice cold 0.15M sodium bicarbonate pH 8, 0.5 M NaCl). The 3 mL swollen beads in coupling buffer were incubated with 1 mL of 5 mg/mL monoclonal in coupling buffer on a rotating mixer overnight at room temperature. It was estimated that 99% of the monoclonal bound to the beads, based on absorbance at 280 nm of the supernatant. Beads were washed with coupling buffer pH 8, water, 1 M KH$_2$PO$_4$ pH 4.2, 1 M TrisCl pH 7.5, water, and phosphate buffered saline (PBS) plus 0.1% (w/v) sodium azide. The washed 3 mL beads were stored in 15 mL PBS, 0.05% azide (w/v). A 0.2 mL aliquot of suspension contained 66 μg monoclonal and a bead volume of 40 μL.

2.5. Immunopurification of BoBChE

BoBChE was immunopurified from 50 mL and 100 mL FBS with mAb2 beads in 0.2 mL and 0.4 mL suspension, and from 380 mL adult bovine serum with mAb2 beads in 2.8 mL suspension. Samples were rotated at 4°C overnight. Beads were washed 12 times with 10 mM TrisCl pH 7.5 until the absorbance at 280 nm of the wash was less than 0.02. The washed beads were assayed for BChE activity. The BChE activity recovered from 50 mL FBS contained 1 unit of BChE activity measured with 1 mM butyrylthiocholine.

2.6. Mass spectrometry analysis of immunopurified BoBChE

Proteins were released from the washed immobilized antibody beads with 50% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid (TFA). The extract was dried, dissolved in 20 μL ammonium bicarbonate pH 8 and digested with trypsin. Tryptic peptides were analyzed by liquid chromatography-tandem mass spectrometry on the 6600 Triple-TOF mass spectrometer (Sciex; Framingham, MA). Details of the mass spectrometry system have been described (Schopfer and Lockridge, 2016).

2.7.1. BChE activity assay—BChE activity was assayed in 0.1 M potassium phosphate pH 7.0 at 25°C with 1 mM butyrylthiocholine (BTC), 0.5 mM 5,5’-dithio-bis-2-nitrobenzoic acid by measuring the rate of increase of absorbance at 412 nm. Units of activity expressed
as μmoles hydrolyzed per min were calculated using an extinction coefficient of 13,600 M$^{-1}$ cm$^{-1}$ (Ellman et al., 1961).

2.7.2. Enzyme linked immunosorbent assay (ELISA) for measuring BChE concentration in bovine serum—The concentration of BChE in adult and fetal bovine sera was estimated by comparing the level of BChE activity captured by mAb2 in ELISA, using pure human BChE as standard. A 96-well Immulon plate was coated with 100 μl of pH 9.6 coating buffer containing 5 μg mAb2 per well. Pure HuBChE was diluted in 1 mg/ml bovine serum albumin (BSA) in Tris buffered saline (TBS) to make a standard set of 2, 3, 4, 5, 7.5, 10, 15, and 20 ng/mL BChE. Adult bovine serum and fetal bovine serum were diluted 1:10 and 1:3 with 1 mg/mL BSA. Eight replicates of each standard and of each diluted bovine serum sample were incubated in the washed, antibody coated wells for 1 h at room temperature. Wells were washed 3 times with TBS containing 0.05% Tween-20. Bound BChE activity was detected by incubating each well with 200 μl of a solution containing 0.5 mM 5,5'-dithio-bis-2- nitrobenzoic acid and 1 mM butyrylthiocholine in 0.1 M potassium phosphate pH 7.0. Intensity of the yellow color was measured at 405 nm in a Bio-Tek Instruments microplate reader after 2 h reaction at room temperature.

2.7.3. Nondenaturing gel electrophoresis stained for BChE and AChE activity—Polyacrylamide 4–30% (v/v) gradient gels with a 4% (v/v) stacking gel were poured in an SE 600, 16 cm vertical slab gel unit (Hoefer Scientific, San Francisco, CA). Gels were run at 320 volts constant voltage for 20 hours at 4°C. Gels stained for activity with BTC or ATC show bands for both BChE and AChE. Gels were stained for activity with either 2 mM BTC iodide or 1.7 mM ATC iodide in a solution containing 90 mL of 0.2 M maleic acid adjusted to pH 6.0 with NaOH, 7.5 mL of 0.1 M sodium citrate, 15 mL of 0.030 M CuSO$_4$ pentahydrate, and 15 mL of 5 mM K$_3$Fe(CN)$_6$ (Karnovsky and Roots, 1964). Reddish brown bands of activity developed in 1–2 hours.

2.8. Inhibition of rBoBChE by chlorpyrifos oxon and spontaneous reactivation
An aliquot of rBoBChE (1.6 units/mL) was inhibited by incubation with an equal volume of chlorpyrifos oxon (CPO) diluted in water to make a final concentration of 0.05 μM CPO in 50 mM TrisCl pH 8.5, 0.25 M NaCl and a final rBoBChE concentration of ~ 0.02 μM. After 30 min incubation at 24°C, activity was inhibited 96%. A 10 μL aliquot of the inhibited rBoBChE mixture was diluted into 2 mL of Ellman reaction mixture containing 1 mM BTC and spontaneous reactivation was monitored by absorbance at 412 nm. The increase in absorbance was recorded for 3.5 hours, as the rBoBChE reactivated.

2.9. Molecular Dynamics modeling
The structure of diethylphosphorylated wild-type human BChE PDB ID: 1XLW (Nachon et al., 2005) solved at 2.10 A resolution was used as the source of atomic coordinates. Residues corresponding to the BoBChE sequence were changed manually and optimized. Water molecules recognized in the crystal structure were included in the model system, and TIP3P water molecules were added, forming a box with boundaries exceeding 10Å from the protein. To make the systems electro-neutral sodium and chloride ions were added up to 0.15 M ion concentration. CHARMM36 force field (Best et al., 2012) was used and the
diethylphosphorylated serine residue was parameterized with CGenFF service (Vanommeslaeghe and MacKerell, 2012; Vanommeslaeghe et al., 2012).

Molecular dynamics (MD) simulations were performed with the NAMD 2.11 program (Phillips et al., 2005) at the Lomonosov Moscow State University supercomputer (Sadovnichy et al., 2013b). During MD simulations, systems were maintained at a constant temperature of 298 K and under pressure of 1 atm (NPT ensemble) by using Langevin dynamics and Nose-Hoover barostat. Periodical boundary conditions and Particle mesh Ewald electrostatics were applied.

Prior to productive MD runs, mutated residues were optimized during 2000 steps of minimization and solvent was equilibrated using a 1 ns equilibrating run with the protein coordinates fixed (except for the mutated ones). After the pre-equilibration 50 ns productive runs were performed for the diethylphosphorylated wild-type HuBChE, the three single mutants (G117S; P285L; F398I), the three double mutants (G117S/P285L; G117S/F398I; P285L/F398I), and the triple mutants (G117S/P285L/F398I and G117H/P285L/F398I) as described (Masson et al., 2013; Lushchekina et al., 2016). The VMD software package (Humphrey et al., 1996) was used for calculation of the solvent-accessible surface area with a 1.4 Å probe radius and analysis of distribution of hydrogen bonds and water molecules in and around the active site.

3. Results

3.1. Comparison of BChE sequences

The amino acid sequence of BoBChE (accession number P32749) is 90% identical to that of HuBChE (accession number P06276). Both have a 28-amino acid signal peptide followed by 574 amino acids for the mature protein (Figure 1). The ATG start site in both genes is surrounded by an unfavorable sequence that does not match the Kozak consensus sequence for initiation of translation.

The quaternary structure of BoBChE is likely to be highly similar to that of HuBChE. The HuBChE structure can be found in Protein Data Bank record 1P0I (Nicolet et al., 2003). Intrachain disulfide bonds at C65-C92, C252-C263, and C400-C519 and the interchain disulfide bond at C571-571 in HuBChE (Lockridge et al., 1987a) are predicted to form in BoBChE. BoBChE has the consensus sequence for 8 Asn-linked glycans, whereas HuBChE has been demonstrated to have 9 Asn-linked glycans (Lockridge et al., 1987b). Residues that form salt bridges in HuBChE are at the same positions in BoBChE (Arg42-Glu90, Arg147-Asp170, Arg265-Glu161, Arg515-Asp395). Forty residues at the C-terminus that constitute the tetramerization domain (Altamirano and Lockridge, 1999) are essentially identical for the two enzymes, except for a Trp to Arg substitution at position 541, where HuBChE has tryptophan and BoBChE has arginine. Both BChE proteins have the catalytic triad residues Ser198, Glu325, His438, the choline binding site Trp82, the peripheral site residues Asp70, Tyr332, and the acyl binding pocket Leu286, Val288, Trp231. The oxyanion hole residue, Gly117 in HuBChE, is Ser117 in BoBChE. See Figure 1. Since all components of a functional BChE are present in BoBChE, it was expected that rBoBChE would have good activity.
3.2. Full-length recombinant BoBChE

Expression levels of full-length rBoBChE were very low for constructs using the native 47- or 28-amino acid signal peptides. The highest expression level of 0.1 units/mL was obtained for the construct containing the 28-residue signal peptide where the ATG start site used the Kozak consensus sequence. Compared to recombinant HuBChE (rHuBChE), the expression level of rBoBChE was 30-fold lower. The expression level of full-length rHuBChE is limited by the quantity of polyproline peptides available for assembly of subunits into tetramers (Larson et al., 2014). It is likely that the low level of rBoBChE is limited by the quantity of the CHO cell-derived polyproline-rich protein that organizes BoBChE subunits into tetramers.

3.3. Recombinant mAb2 expressed in culture medium extracts BoBChE from 10% (v/v) FBS

The nucleotide and deduced amino acid sequence of monoclonal mAb2 were determined by DNA sequencing of the PCR product (Peng et al., 2015). This strategy leaves open the possibility that the PCR-amplified DNA contains errors. To confirm that the sequence is correct it is important to compare the characteristics of the recombinant monoclonal to that of the monoclonal purified from ascites fluid. When recombinant mAb2 was expressed in HEK293 cells in culture medium containing 10% (v/v) FBS, the purified monoclonal antibody had BChE activity, indicated by a band at the very top of the gel attributable to the BoBChE-mAb2 complex (Figure 2A lane 3). Note that FBS contains both AChE and BChE, however mAb2 selectively bound BoBChE (Figures 2B and 2C) and did not bind BoAChE. The presence of BoBChE activity in the purified monoclonal antibody means that the recombinant mAb2 expressed in HEK293 cells captured BoBChE from the culture medium and held on to it at pH 2.6 while the monoclonal was purified on Protein A agarose. This observation confirms that the sequences used for expression of mAb2 represent the desired monoclonal. Furthermore, this result shows that FBS contains BoBChE and that mAb2 binds BoBChE. 14

3.4. BoBChE binds to anti-human BChE monoclonal mAb2 while BoAChE does not

In contrast to recombinant mAb2 purified from culture medium containing 10% (v/v) FBS, mAb2 purified from mouse ascites fluid had no BChE activity (Figure 2A lane 2). Mouse ascites fluid contains mouse BChE (Figure 2A lane 1), but mouse BChE does not bind to monoclonal mAb2 produced in mouse (Peng et al., 2016a), thus yielding a monoclonal free of BChE activity.

FBS contains both AChE and BChE. BoBChE and BoAChE both hydrolyze acetylthiocholine (ATC) and butyrylthiocholine (BTC). BoBChE has higher activity with BTC than with ATC, while BoAChE has higher activity with ATC than BTC. The activity of concentrated rBoBChE was 1.1 units/mL with 1 mM BTC and 0.4 units/mL with 1 mM ATC. Thus, rBoBChE hydrolyzed 1 mM BTC 3-fold more rapidly than 1 mM ATC. Conversely, purified BoAChE hydrolyzed 1 mM ATC 80-fold more rapidly than 1 mM BTC at pH 7.0. Figure 2B lane 5 in the gel stained with BTC shows that FBS has a band for BoBChE activity near the top of the gel and a weak band for BoAChE further down. Demonstration that the band near the top of the gel is BoBChE comes from experiments in

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which monoclonal mAb2 from ascites fluid immobilized on Sepharose beads was incubated with FBS. The beads captured BoBChE from FBS. The unbound cholinesterases were visualized in the gel stained with BTC in Figure 2B. The BoBChE band near the top of the gel disappeared (see Figure 2B, lanes 6 and 7), whereas the BoAChE band intensities remained unchanged. Similarly, in Figure 2C in the gel stained with ATC, the weak band for BoBChE near the top of the gel disappeared following treatment with mAb2 (Figure 2C lanes 10 and 11), whereas the intensities of BoAChE bands were unaffected by mAb2. It was concluded that FBS contains BoBChE and BoAChE, and that mAb2 captures BoBChE, but not BoAChE.

The finding that mAb2 selectively extracted BoBChE from FBS allowed us to immunopurify BoBChE. However, binding BoBChE to immobilized monoclonal mAb2 could not be used to isolate active BoBChE because the BoBChE was not released from the antibody by mild conditions, e.g. with pH 11 or pH 2.6 buffers or with 3 M NaCl. Inactive BoBChE could be released with 50% (v/v) acetonitrile, 1% (v/v) TFA, but this solvent denatured the enzyme. The denatured BoBChE was suitable for mass spectrometry analysis.

A second anti-human BChE monoclonal, B2 18-5, which like mAb2 has broad species specificity (Peng et al., 2016a), did not bind to either BoBChE or BoAChE.

3.5. Mass spectrometry evidence for BChE in fetal bovine serum

Peptides representing 47% of the BoBChE protein were identified by Protein Pilot analysis of mass spectral data for tryptic peptides from BoBChE immunopurified from 50 and 100 mL FBS (Figure 3). This positive identification provided conclusive proof that FBS contains BoBChE.

3.6. Mass spectrometry evidence for BChE in adult bovine serum

Peptides representing 16% of BoBChE were identified in BChE immunopurified from 380 mL of adult bovine serum. The low recovery of BoBChE from adult bovine serum is consistent with the conclusion that adult bovine serum contains very low levels of BChE.

Our mass spectrometry data provided no evidence for the presence of polyproline peptides in BoBChE, such as those in soluble BoAChE tetramers (Biberoglu et al., 2013). We hypothesize that in place of short polyproline peptides, BoBChE tetramers contain a polyproline-rich protein, but at this time we do not know the identity of the tetramer-organizing protein in BoBChE.

3.7. BoBChE tetramers are larger than HuBChE tetramers

In Figure 4 (lanes 3, 4, 10, 11) BoBChE is at the top of the 4–30% (v/v) gradient gel. In contrast, HuBChE tetramers with a molecular weight of 340,000 Da migrate well into the gel toward the positive electrode (Figure 4 lanes 1, 2, 7, 8, 9, 14). The 90% identity between bovine and human BChE led to the expectation that BoBChE tetramers would have a mass of about 340,000 Da. The top-of-the-gel position for BoBChE indicates the molecular weight of the BoBChE tetramer is actually much greater than that of the HuBChE tetramer. The greater mass suggests that BoBChE is in complex with another protein.
Recombinant human BChE (rHuBChE) expressed in CHO cells assembles into 340,000 Da tetramers using polyproline-rich peptides of CHO cell origin (Schopfer and Lockridge, 2016). These tetramer-organizing peptides range in size from 6 to 41 amino acids. We therefore expected that rBoBChE expressed in CHO cells would assemble into 340,000 Da tetramers by incorporating polyproline peptides contributed by CHO cells. Unexpectedly, full-length rBoBChE migrated to the same top-of-the-gel position as BoBChE from FBS (Figure 4 lanes 5, 6, 12, 13). This result suggests that BoBChE subunits are not organized into tetramers by interaction with short polyproline peptides. It seems that BoBChE subunits assemble into tetramers by interaction with a large protein whose identity is unknown at this time.

3.8. Kinetic characterization of rBoBChE

Full-length rBoBChE expressed by CHO cells into serum-free Ultraculture was partially purified by anion exchange chromatography on Q Sepharose fast flow. The rBoBChE preparation had an activity of 1.6 units/mL with 1 mM BTC in pH 7 buffer at 25°C.

Dependence of the rBoBChE catalyzed steady state turnover of BTC on BTC concentration yielded a substrate activation profile for BTC concentrations between 8 μM and 30 mM. This is similar to the substrate dependence of velocity found for HuBChE which also exhibits substrate activation (Masson et al., 2001). Data were fit to the substrate activation model of Radic (Radic et al., 1993) using the Solver routine from Microsoft Excel. The concentration of rBoBChE was determined by titration with chlorpyrifos oxon and used to calculate $k_{cat}$ from $V_{max}$. The $K_m$ and $b$ values for rBoBChE are similar to those for HuBChE, however, $k_{cat}$ for rBoBChE is about twice as large as $k_{cat}$ for HuBChE (Table 1). The higher $k_{cat}$ for rBoBChE may be rationalized by substantial differences between the active sites of rBoBChE and HuBChE, notably G117S in the oxyanion hole, P285L in the acyl-binding pocket, and F398I also in the acyl-binding pocket. The latter two mutations should create more space in the active site pocket (see the Molecular Dynamics calculations of the BChE active site section below).

The BChE-specific inhibitor ethopropazine inhibited rBoBChE with a $K_i$ of 2.2 μM, which is 10-fold weaker than for HuBChE (Table 1). The $K_i$ value was measured using 0.05 to 2 mM BTC and 0 to 15 μM ethopropazine.

3.9. Concentration of AChE and BChE in bovine serum

The concentration of BoAChE was estimated to be 0.55 μg/mL in FBS and 0.05 μg/mL in adult bovine serum (Table 2). These values were calculated using a specific activity of 2000 units/mg for BoAChE measured at pH 7.0 with 1.0 mM ATC. AChE concentrations of 0.6 μg/mL and 1.4 μg/mL in FBS were reported for BoAChE purified from 44,000 mL and 250,000 mL of FBS (Ralston et al., 1985; De la Hoz et al., 1986). The concentration of BoBChE was estimated from ELISA using pure HuBChE as standard.

3.10. Spontaneous reactivation of rBoBChE inhibited by chlorpyrifos oxon

Chlorpyrifos oxon (CPO) is the active metabolite of the pesticide, chlorpyrifos. Treatment of 0.011 μM rBoBChE with 0.05 μM CPO inhibited 96% of the rBoBChE activity in 30 min.
At this point, a 10 μl aliquot from the inhibition mixture was diluted 200-fold into a 2 mL activity assay containing 1 mM BTC. The inhibited rBoBChE spontaneously recovered some activity. The amount of active rBoBChE increased for 140 min at which time 14% of the original activity had returned (see Figure 5). After 140 min BTC hydrolysis continued linearly and was followed to 225 min. The 200-fold dilution of CPO plus the presence of 1 mM BTC in the activity assay strongly argues that re-inhibition of rBoBChE by residual CPO is not the cause of the incomplete recovery of activity. This suggests that the partial restoration of activity is a consequence of competition from irreversible aging.

The first-order approach to steady state shown in Figure 5 is described by equation 1:

\[ P = (V_{\text{max}}) t - \frac{V_{\text{max}}}{k} + \frac{V_{\text{max}}}{k} e^{-kt} + \text{background} \]

Equation 1

where \( P \) is product; \( V_{\text{max}} \) is the maximum rate for BTC turnover by the recovered rBoBChE; \( k \) is the observed first order rate for recovery of activity; and \( t \) is time (Hatfield et al., 1970). When the first order recovery phase is complete, the resultant steady state rate is described by equation 2:

\[ P = (V_{\text{max}}) t - \frac{V_{\text{max}}}{k} + \text{background} \]

Equation 2

The steady state slope and intercepts from Figure 5 yielded values for \( V_{\text{max}} \) of 0.0056 ΔA412/min and for background of 0.0825 ΔA412. Introducing these values into equation 2 yields a value for reactivation, \( k = 0.0161 \text{ min}^{-1} \). Because the recovery process is an irreversible bifurcation of the inhibited rBoBChE between dephosphorylation and aging, the observed rate for reactivation (\( k \)) is equal to the sum of the rates for dephosphorylation (\( k_r \)) and aging (\( k_a \)); and the ratio of the amount of dephosphorylated enzyme to the amount of aged enzyme equals the ratio of \( k_r/k_a \) (Frost and Pearson, 1965). The uninhibited rate of turnover (0.0388 ΔA412/min) is proportional to the total amount of rBoBChE while the recovered rate of turnover (0.0056 ΔA412/min) is proportional to the amount of dephosphorylated rBoBChE. Using these values, the ratio \( k_r/k_a \) can be calculated (0.1687). From the ratio of rates and the sum of rates (0.0161 min\(^{-1}\)), a value for the rate of dephosphorylation of 0.0023 min\(^{-1}\) and a value for the rate of aged adduct formation of 0.0138 min\(^{-1}\) were calculated (Table 3).

3.11. Molecular dynamics simulations of the BChE mutants

Molecular dynamics (MD) simulations were performed on CPO-inhibited “bovinated” HuBChE with replacements from the BoBChE sequence: for the three single mutants (G117S; P285L; and F398I), the three double mutants (G117S/P285L; G117S/F398I; and P285L/F398I), and the triple mutants (G117S/P285L/F398I and G117H/P285L/F398I). CPO-inhibited HuBChE is diethoxyphosphorylated on Ser198. Results showed that in the triple mutant accessibility of the diethoxyphosphate phosphorus atom to a water molecule is significantly increased (Figure 6) as compared to wild-type HuBChE and double mutants. The average solvent accessible surface area for the Ser198+CPO conjugate is 423±7 A\(^2\) for

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wild-type HuBChE, 420±9 Å² for G117S/F398I and 431±8 Å² for the G117S/P285L/F398I mutant.

Accessibility of the phosphorus atom to water is a critical factor in the spontaneous dephosphorylation reaction. MD simulations reveal that all three mutations play a role in spontaneous reactivation of BoBChE. F398I creates space for a water molecule in the vicinity of the phosphorus atom. The water molecule collides with the F398 surface, but has no steric conflict with I398 (Figure 7). P285L increases water accessibility around the diethoxyphosphate residue because the longer side chain of leucine interacts with neighboring residues, Y332 and F357, which keeps it away from the active site (left part of Figure 7). G117S acts in a different way. The area around the diethoxyphosphate adduct is highly hydrophobic in the P285L/F398I mutant. Mutation G117S makes the area more hydrophilic, and the hydration shell of S117 serves as a source of water molecules for the dephosphorylation reaction. This also would accelerate deacylation of the intermediate in substrate hydrolysis. In addition, S117 forms a hydrogen bond with one of the two ethoxy groups of diethoxyphosphate (Figure 7). This H-bond stabilizes the conjugate and could play a role in modulating the energetics of the dephosphorylation (reactivation) and the dealkylation (aging) reactions. Taken together, results of MD simulations suggest that these three mutations in the BoBChE sequence are responsible for increasing spontaneous dephosphorylation of the CPO-inhibited enzyme, and the increased kcat with BTC as substrate compared to HuBChE. However, these features of the BoBChE active site are not sufficient to produce the fast reactivation that is observed in the G117H mutant of HuBChE. A critical difference between G117H and G117S is that H117 is capable of becoming protonated on the imidazole ring. Nachon et.al have argued that the protonated form of H117 would enhance the electrophilicity of the phosphorus atom by local electrostatic effects, thereby promoting dephosphorylation (Nachon et al., 2011), which was later supported by QM/MM calculations (Masson and Lushchekina, 2016).

4. Discussion

4.1. Why is the apparent molecular weight of BoBChE so high?

The 4 subunits of plasma HuBChE are held together by polyproline-rich peptides 20–32 residues in length that derive from the Ras-associated and pleckstrin homology domain-containing protein, lamellipodin (Li et al., 2008; Peng et al., 2016b). The polyproline peptides add a mass of 2 to 3 kDa to the 340 kDa tetramer. Because the C-terminal tetramerization domain of BoBChE is essentially identical to the C-terminal tetramerization domain of HuBChE, we expected that BoBChE would form tetramers using a strategy involving polyproline-rich peptides, similar to that for HuBChE. However, to date we have no mass spectrometry evidence for the presence of polyproline peptides in BoBChE tetramers. To account for the increased mass of BoBChE tetramers, we hypothesize that BoBChE tetramers include a large polyproline-rich protein in place of the 2 to 3 kDa polyproline peptides found in HuBChE tetramers. Models of the human BChE tetramer containing a short polyproline peptide and the bovine BChE tetramer containing a large polyproline-rich protein are shown in Figure 8.
We have found that rHuBChE tetramers expressed in CHO cells contain polyproline-rich peptides that derive from a variety of CHO cell proteins (Schopfer and Lockridge, 2016). Thus rHuBChE appears to use polyprolines obtained adventitiously. The sizes of rHuBChE tetramers and plasma HuBChE tetramers are an identical 340,000 Da. We expected that rBoBChE tetramers would use the same CHO-cell polyprolines that we found in rHuBChE. However, rBoBChE, like native plasma BoBChE, is much larger than HuBChE. This argues that the tetramer-forming polyproline peptides used by HuBChE are not suitable for BoBChE. This in turn suggests that tetramer-formation for BoBChE requires a specialized polyproline-rich protein.

The conclusion that BoBChE has a large size relies on the observation that BoBChE migrates slowly toward the positive electrode on a nondenaturing polyacrylamide gel. An alternative explanation for slow migration would be a high isoelectric point, with a net positive charge. Each BoBChE subunit contains 60 negatively charged residues, 63 positively charged residues, and the consensus site for 8 N-linked glycans terminating in 16 negatively charged sialic acids. The glycans are located on the surface of the protein. The large negative charge contributed by the glycans rules out the likelihood that positive charge rather than large size explains the slow migration of BoBChE on a nondenaturing polyacrylamide gel. The presence of consensus sites for 8 N-linked glycans on BoBChE suggests, but does not prove the sites are occupied. If deglycosylated, the BChE would aggregate into large particles that barely enter a gel.

4.2. Low concentration of BChE in bovine serum

We speculate that the low concentration of BChE in bovine serum is explained by two factors: 1) the unfavorable nucleotide sequence at the ATG start site, and 2) limiting quantities of the proposed polyproline-rich protein that organizes subunits into BoBChE tetramers. The polyproline-rich peptides in HuBChE derive from lamellipodin (Li et al., 2008; Peng et al., 2016b). The identity of the proposed polyproline-rich protein in BoBChE is not yet known.

4.3. All ruminants have very low serum BChE activity

The very low BChE activity in the sera of other ruminants may have the same explanation as the low BChE activity in bovine serum. Our studies have demonstrated that the quantity of rHuBChE tetramers secreted into culture medium is limited by the availability of polyproline-rich peptides (Larson et al., 2014). We hypothesize that the concentration of ruminant BChE secreted into serum is low because only a limited amount of the 32 appropriate polyproline-rich protein is available for organizing subunits into stable tetramers.

4.4. Use of transgenic cows to produce human BChE

We estimate that adult bovine serum has a BChE concentration of about 0.03 μg/mL (Table 2). In contrast, adult human serum has a concentration of 4 to 5 μg/mL. The low concentration of BChE in adult cow serum recommends the cow as a suitable host for production of HuBChE by recombinant DNA methods. Native BoBChE is easily separated from HuBChE on procainamide and hupresin affinity columns because HuBChE binds to
these affinity gels, but BoBChE does not. However, the structure of glycans on the rHuBChE protein produced in transgenic cows will differ from that in native HuBChE (van Berkel et al., 2002). The non-human glycan structures are likely to induce an immune response in humans treated with rHuBChE produced in cows.

4.5. Spontaneous reactivation

Reactivation of CPO inhibited BChE involves dissociation of diethoxyphosphate from the active site serine. Dephosphorylation of the diethoxyphosphorylated G117H mutant of HuBChE at a rate of 1.2 min\(^{-1}\) is 60,000-fold faster than the rate for wild-type HuBChE (Table 3), implicating the amino acid at position 117 in the dephosphorylation process. The rate for dephosphorylation of diethoxyphosphorylated rBoBChE (0.0023 min\(^{-1}\)) is 2-orders of magnitude faster than the rate for dephosphorylation of wild-type diethoxyphosphorylated HuBChE (Table 3). From the amino acid sequence in Figure 1, it can be seen that rBoBChE carries a serine in place of glycine at position 117. It is tempting to propose that the increased rate for dephosphorylation of the diethoxyphosphorylated rBoBChE is due simply to this serine in position 117. However, to a first approximation, the G117S mutant of HuBChE does not undergo spontaneous dephosphorylation after diethoxyphosphosphate inhibition (Schopfer et al., 2004). This observation is supported by QM/MM calculations on the G117H HuBChE mutant which show that the protonated form of His117 stabilizes the intermediate and lowers the energy barrier of the dephosphorylation reaction, an effect which cannot be achieved by a non-ionizable serine in this position (Masson and Lushchekina, 2016). This, in turn, argues that factors in addition to the nature of the residue at position 117 significantly influence the dephosphorylation of diethoxyphosphorylated BoBChE. In addition to the G117S mutation, BoBChE has two residues near the active center, L285 and I398, that differ from P285 and F398 in HuBChE (Figure 1). Residues L285 and I398 in BoBChE allow a water molecule access to the diethoxyphosphate-serine in the active site. The water molecule can assume a position to attack the phosphorus atom, thus releasing diethoxyphosphate from the active site serine and reactivating the BoBChE enzyme. The higher k\text{cat} of BoBChE for hydrolysis of BTC compared to HuBChE can also be explained by this feature.

4.6. Monitoring pesticide exposure of cattle

BChE in human plasma is a sensitive biomarker of exposure to organophosphorus pesticides and nerve agents (Fidder et al., 2002; Sporty et al., 2010; Carter et al., 2013; Pantazides et al., 2014). However, the low concentration of BChE in bovine plasma makes BoBChE an unreliable indicator of exposure in cattle. Since 90% of the cholinesterase activity in bovine whole blood is due to AChE in erythrocytes, it was recommended to measure AChE activity in red blood cells for assessing exposure of cattle to cholinesterase-inhibiting pesticides (Pardio et al., 2001).

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Abbreviations

- **AChE**: acetylcholinesterase
- **ATC**: acetylthiocholine iodide
- **BChE**: butyrylcholinesterase
- **BoBChE**: bovine butyrylcholinesterase
- **rBoBChE**: recombinant bovine butyrylcholinesterase
- **BoAChE**: bovine acetylcholinesterase
- **BSA**: bovine serum albumin
- **BTC**: butyrylthiocholine iodide
- **CHO**: Chinese Hamster Ovary cells
- **CPO**: chlorpyrifos oxon
- **ELISA**: enzyme linked immunosorbent assay
- **FBS**: fetal bovine serum
- **HEK293**: human embryonic kidney cells
- **HuBChE**: human butyrylcholinesterase
- **mAb2**: anti-human BChE monoclonal
- **MD**: molecular dynamics
- **PBS**: phosphate buffered saline
- **rHuBChE**: recombinant human butyrylcholinesterase
- **TBS**: tris buffered saline
- **TFA**: trifluoroacetic acid
- **QM/MM**: quantum mechanics/molecular mechanics

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• Bovine serum contains very low quantities of butyrylcholinesterase (BChE).
• Fetal bovine serum has 10 times more BChE than adult bovine serum.
• Bovine BChE tetramers are much larger than 340 kDa.
• Bovine BChE inhibited by chlorpyrifos oxon spontaneously reactivates.
• Monoclonal mAb2 immunopurifies bovine BChE.
Figure 1.
Comparison of amino acid sequences of human and bovine BChE. Residues in the catalytic triad are Ser 198, Glu 325, and His 438. Boxed residues Ser 117, Leu 285 and Ile 398 are implicated in spontaneous reactivation of BoBChE inhibited by chlorpyrifos oxon.
Figure 2.
Nondenaturing gradient gel stained with butyrylthiocholine (BTC) or acetylthiocholine (ATC). Lane 1, mouse ascites fluid containing mAb2; lane 2, mAb2 purified from ascites fluid; lane 3, recombinant mAb2 purified from HEK293 culture medium containing 10% (v/v) FBS; lanes 4 and 5, FBS; lanes 6 and 7, FBS after treatment with mAb2 from ascites; lanes 8 and 9, FBS; lanes 10 and 11, FBS after treatment with mAb2 from ascites.
Mass spectrometry identified BoBChE (P32749) in FBS. Peptides colored green were identified with ≥95% confidence. Peptides colored yellow were identified with 50–95% confidence.
Figure 4.
Nondenaturing gradient gels stained for cholinesterase activity with ATC or BTC. Human plasma (lanes 1, 2, 8, 9) has an intense band of BChE activity for the C4 tetramer. Minor HuBChE components C1, C2, and C3 are more easily visualized by staining with BTC. FBS has an intense band for BoAChE tetramers in the gel stained with ATC (lanes 3, 4) and a weak AChE band in the gel stained with BTC (lanes 10, 11). BoBChE in FBS (lanes 3, 4, 10, 11) is the band at the top of the gel. The most intense band for rBoBChE is at the top of the 19 gel (lanes 5, 6, 12, 13). Pure HuBChE tetramers (lanes 7, 14) migrate to the same position as BChE tetramers in human plasma.
Figure 5.
Spontaneous reactivation of CPO-inhibited rBoBChE.
Figure 6.  
Fraction of snapshots from the 50 ns MD trajectory with a water molecule found within 3.5Å of the phosphorus atom from the diethoxyphosphate adduct of S198, for different mutants of HuBChE. The triple mutants allow water to have greater access to the phosphorus atom.
Figure 7.
Model to explain spontaneous reactivation of BoBChE inhibited by chlorpyrifos oxon. The model is based on the crystal structure of diethoxyphosphorylated HuBChE (1XLW). Carbon atoms in the mutated residues G117S/P285L/F398I are light blue. Carbon atoms in native HuBChE residues are green. The CPO (diethoxyphosphate) bound serine 198 and a water molecule are shown as balls and sticks. Residue F398 in HuBChE is shown as a semi-transparent surface. Residue I398 in BoBChE creates space for a water molecule near the phosphorus molecule (orange). Mutation P285L increases water access to the phosphorus atom because L285 in BoBChE, but not P285 in HuBChE, points away from the active site Ser 198. The orientation of L285 is stabilized by interaction with Y332 and F357. Residues Y332 and F357 are the same in HuBChE and BoBChE. The structure was drawn with PyMol.
Figure 8.
Models of the human and bovine BChE tetramers. Panels A and B are top and side views of the human BChE tetramer, showing the location of a short polyproline peptide within the C-terminal tetramerization domain (Reproduced from Pan Y, Muzyka JL, Zhan CG. J Phys Chem 2009; 113, 6543-52). Panels C and D are top and side views of the bovine BChE tetramer showing the location of a large polyproline-rich protein within the tetramerization domain and extending beyond the plane of the 4 subunits. This model was developed for the collagen-tailed AChE tetramer by Dvir et al. (Reproduced from Dvir H, Harel M, Bon S, Liu WQ, Vidal M, Garbay C, Sussman JL, Massoulie J, Silman I. EMBO J 2004; 23, 4394–405).
### Table 1

Steady state turnover of BTC by rBoBChE and inhibition of BTC turnover by ethopropazine

|                | Km, μM | Kss, mM | kcat, min⁻¹ | b  | bKcat, min⁻¹ | Ki ethopropazine, μM | ref                                |
|----------------|--------|---------|--------------|----|--------------|----------------------|-----------------------------------|
| rBoBChE        | 35     | 500     | 54,100       | 1.9| 103,000      | 2.2                  | Present work                      |
| HuBChE         | 20–28  | 1.5     | 25,000–27,000| 3.1–3.4| 80,000–90,000| 0.16; 0.2             | (Saxena et al., 1997; Masson et al., 2001; Sinko et al., 2011) |
Table 2
Estimated concentrations of AChE and BChE in bovine serum

| Serum          | AChE activity, units/mL | [AChE] μg/mL | BChE activity, units/mL | [BChE] μg/mL |
|----------------|-------------------------|--------------|-------------------------|--------------|
| FBS            | 1.1                     | 0.55         | 0.03                    | 0.04         |
| Adult bovine   | 0.09                    | 0.05         | 0.02                    | 0.03         |
### Table 3
Dephosphorylation and aging of diethoxyphosphate-modified HuBChE, rHuBChE

| Enzyme             | Residue at position 117 | Rate of dephosphorylation, min⁻¹ | Rate of aging, min⁻¹ | reference                                      |
|--------------------|-------------------------|----------------------------------|----------------------|------------------------------------------------|
| HuBChE             | Gly                     | 0.000015 (37°C)                  | --                   | (Davison, 1955)                                  |
| HuBChE             | Gly                     | --                               | 0.00096 (37°C) (t₁/₂=722 min⁻¹) | (Mason et al., 1993; Masson et al., 1997)        |
| rHuBChE, G117H     | His                     | 1.2 (t₁/₂=0.6 min⁻¹)             | 0.002 (t₁/₂=346 min⁻¹) | (Lockridge et al., 1997; Schopfer et al., 2004) |
| rHuBChE, G117S     | Ser                     | 0                                | --                   | (Schopfer et al., 2004)                          |
| rBoBChE            | Ser                     | 0.0023 (t₁/₂=301 min⁻¹)          | 0.0138 (t₁/₂=50 min⁻¹) | This work                                      |

*a This value is for the measured rate of reactivation, which is the sum of the rates for dephosphorylation and aging.