Title

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Journal

Biotechnology and Bioengineering, 115(5)

ISSN

0006-3592

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Publication Date

2018-05-01

DOI

10.1002/bit.26557

Peer reviewed

HHS Public Access

Author manuscript

Biotechnol Bioeng. Author manuscript; available in PMC 2020 July 08.

Published in final edited form as:

Biotechnol Bioeng. 2018 May ; 115(5): 1301–1310. doi:10.1002/bit.26557.

Purification, characterization, and N-glycosylation of recombinant butyrylcholinesterase from transgenic rice cell suspension cultures

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Abstract

Recombinant butyrylcholinesterase produced in a metabolically regulated transgenic rice cell culture (rrBChE) was purified to produce a highly pure (95%), active form of enzyme. The developed downstream process uses common manufacturing friendly operations including tangential flow filtration, anion-exchange chromatography, and affinity chromatography to obtain a process recovery of 42% active rrBChE. The purified rrBChE was then characterized to confirm its comparability to the native human form of the molecule (hBChE). The recombinant and native enzyme demonstrated comparable enzymatic behavior and had an identical amino acid sequence. However, rrBChE differs in that it contains plant-type complex N-glycans, including an α-1,3 linked core fucose, and a β-1,2 xylose, and lacking a terminal sialic acid. Despite this difference, rrBChE is demonstrated to be an effective stoichiometric bioscavenger for five different organophosphorous nerve agents in vitro. Together, the efficient downstream processing scheme and functionality of rrBChE confirm its promise as a cost-effective alternative to hBChE for prophylactic and therapeutic use.

Keywords

butyrylcholinesterase; downstream processing; Hupresin; N-glycosylation

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CONFLICTS OF INTEREST

Karen A. McDonald is a co-founder of Inserogen, Inc., a plant-based biotechnology company with a focus on the development of orphan drugs for replacement therapy. The data analyses, results presented, and outcomes of this study are personal views of independent authors and do not reflect any financial or commercial interest of either the USAMRICD or UC Davis.

1 | INTRODUCTION

For decades, the human enzyme butyrylcholinesterase (BChE, EC 3.1.1.8) has been studied as a therapeutic and prophylactic treatment against organophosphate poisoning and cocaine toxicity (Lockridge, 2015). However, its widespread use has been limited by the low availability and limited abundance of human BChE from human blood plasma: using current technologies, the estimated cost for a single treatment dose of plasma-derived BChE is ~US $20,000 (DARPA, 2012). The production of recombinant BChE in a variety of expression systems (Alkanaimsh et al., 2016; Brazzolotto et al., 2012; Chilukuri et al., 2005; Corbin et al., 2016; Geyer et al., 2010; Huang et al., 2007; Ilyushin et al., 2013; Schneider, Castilho et al., 2014) has demonstrated the possibility of high level production of active enzyme, but the combined costs of production and purification are still prohibitively high.

Our group has recently demonstrated production of active, rice recombinant BChE (rrBChE) from a metabolically regulated transgenic rice (*Oryza sativa*) cell culture (Corbin et al., 2016). This platform presents a promising method for cost-effective production of BChE due to the low costs of plant cell culture media and the ability to operate the culture semicontinuously over long (>3 month) production runs. To build upon this work, we have developed a downstream processing scheme that uses common, scalable bioprocessing methods to obtain a high purity, therapeutic-quality product. This creates additional opportunity for cost-savings since the process makes use of standard, widely available unit operations including tangential flow filtration (TFF), diethylaminoethanol (DEAE) anion-exchange chromatography, and affinity chromatography. Use of such operations removes the need for custom equipment, specialized supplies, or significant reconfiguration of existing facilities developed for other cell-based manufacturing scale processes used today.

Historically, procainamide affinity resin has been the predominant tool used to purify both native and recombinant BChE and acetylcholinesterase (AChE) (Lockridge & La Du, 1978). It has been successfully used to purify human BChE from plasma (Grunwald et al., 1997; Lockridge, Schopfer, Winger, & Woods, 2005; Mehrani, 2004; Saxena, Tipparaju, Luo, & Doctor, 2010) and Cohn fraction IV (Lynch et al., 1997; Saxena, Luo, & Doctor, 2008; Saxena et al., 2010), as well as recombinant BChE and/or AChE from diverse expression platforms including from the milk of transgenic goats (Huang et al., 2007), transgenic plants (Geyer et al., 2010), HEK 293 cell culture media (Chilukuri et al., 2005), and several others. In most reported data, the procainamide resin is preceded by an initial step to enrich the protein pool for the BChE, including PEG (Chilukuri et al., 2005) and ammonium sulfate (Chilukuri et al., 2005; Geyer et al., 2010) precipitation or a capture chromatography step, including Concanavalin A (Geyer et al., 2010) and DEAE (Lynch et al., 1997; Mehrani, 2004) chromatography. Some studies also reported a final polishing step, such as size exclusion (Ilyushin et al., 2013) or DEAE (Lockridge et al., 2005; Saxena et al., 2010) chromatography.

Despite procainamide's ability to purify from multiple expression systems, the overall yield of procainamide-based purification is varied, but typically low. Most reported research has not quantified the final purification yield. From human plasma or Cohn fraction IV, overall yields range from 20% to 50% (Kaya, Özcan, Şişecioğlu, & Ozdemir, 2013; Ralston, Main,

Kilpatrick, & Chasson, 1983; Saxena et al., 2008, 2010). The only reported yield from a crude cell extract in a recombinant expression system was for transgenic *Nicotiana benthamiana* plants, for which an overall process recovery of 13% was reported (Geyer et al., 2010). A few notable exceptions to these lowyields include reports of a 59%yield of hBChE purification from plasma (Grunwald etal., 1997) and a 72% yield of recombinant BChE secreted from CHO cell culture (Ilyushin et al., 2013). These exceptions can be explained in part by the fact that procainamide has been known to have a binding capacity that varies with the purity of the sample loaded onto the column: when the sample is already 50–70% pure, procainamide resin has a binding capacity of 3 mg BChE per millilitre of resin. However, at low purities (<0.1%) of BChE, the capacity is on the order of 10 μg BChE per millilitre of resin. This is due to a high incidence of non-specific binding of other proteins to the resin (Lockridge et al., 2005). These two studies that report high BChE yields had high initial purities of BChE, and utilized techniques that are not preferred for industrial scale processes such as long resin contact times, ammonium sulfate fractionation, and size exclusion chromatography. Therefore, to meet the need for a cost-effective platform for production and purification of BChE, yields from these downstream processes must be improved, but in a manner that is rapid, linearly scalable, and relatively inexpensive.

To address this problem, developing an improved affinity resin for BChE has been a major point of focus. The most successful improvement was the development of Hupresin (Brazzolotto et al., 2012), which is composed of a derivative of a potent cholinesterase inhibitor called huprine X (Ronco et al., 2011) grafted to a sepharose chromatography scaffold. Brazzolotto et al. demonstrated Hupresin's efficacy in the purification of recombinant BChE secreted to the medium of insect cell culture. Though no process yield was reported, a >90% purity of the product was achieved in a single chromatography step, while procainamide resin requires multiple process steps to reach the same purity level. In this work, we provide the first report of Hupresin-based purification of active BChE from a plant cell culture system.

The ability to effectively purify rrBChE enables us to characterize the molecule and demonstrate that this recombinant enzyme is structurally and functionally comparable to native human BChE (hBChE). We also determine the structure of the plant-made N-glycans present on the molecule. While there has been no definitive evidence of immunogenicity of plant-made glycans (Shaaltiel & Tekoah, 2016), it is important to understand these structures as they may impact the activity, biodistribution, or pharmacokinetic stability of the molecule. Finally, we can compare the inhibition of rrBChE and hBChE by several organophosphate (OP) molecules to demonstrate rrBChE's in vitro efficacy as nerve agent bioscavenger.

2 | MATERIALS AND METHODS

2.1 | rrBChE expression and extraction from rice cell culture

Suspension cultures of rice callus were grown and induced in a 5 L bioreactor according to the methods previously described (Corbin et al., 2016). Cultures were grown for 7 days prior to induction via sugarstarvation, then cultured for 5 days for rrBChE production before the biomass was harvested and extracted. Biomass was first separated from the culture medium via vacuum filtration using Whatman 1 filters (GE Healthcare Life Sciences, Marlborough,

MA). Crude cell extracts were obtained by grinding the callus in a 1:1 (fresh weight:volume) ratio in cold (4 °C) homogenization buffer (20 mM sodium phosphate, pH 7.4). The homogenized sample was then clarified via centrifugation at 4 °C. The clarified solution was filtered through 1.2, 0.45, and 0.22 μm pore size filters to obtain the sterile crude extract.

2.2 | Ultrafiltration and diafiltration of the extract

Microfiltered crude extracts were concentrated and diafiltered against 20 mM sodium phosphate buffer, pH 7.4 using a 30kDa Minimate Tangential Flow Filtration (TFF) Capsule (Pall Corporation, Ann Arbor, MI) and an ÄKTA Flux s TFF unit (GE Healthcare Life Sciences). First, the optimal transmembrane pressure (TMP) for ultrafiltration was selected by determining the relationship between TMP and permeate flux for the extract. Using this TMP, the crude extract was concentrated 10-folds before continuously diafiltering with four sample volumes of 20 mM sodium phosphate buffer, pH 7.4.

2.3 | Anion exchange column chromatography

The concentrated extract was filtered through a 0.22 μm filter and loaded onto a DEAE FF HiScreen prepacked column and purified using an ÄKTA Pure chromatography unit (both from GE Healthcare Life Sciences). The column was both equilibrated and washed with the loading buffer (20 mM phosphate buffer, pH 7.4). Adsorbed proteins were eluted isocratically with 300 mM NaCl over five column volumes, and the elution fractions showing the highest BChE activity were pooled to prepare for affinity chromatography. Both sample loading and elution were performed at 1 ml/min.

2.4 | Hupresin chromatography

Pooled elution fractions from DEAE chromatography were purified using Hupresin (CHEMFORASE, Rouen, France). The resin was packed into an XK16/20 column (GE Healthcare Life Sciences) and operated on an AKTA Pure chromatography unit. The column was equilibrated and washed with 20 mM phosphate buffer, 150 mM NaCl, pH 7.4, then eluted isocratically with 500 mM tetramethylammonium chloride (TMAC) over five column volumes, all at 1 ml/min.

2.5 | Gel electrophoresis and immunoblotting

SDS-PAGE was performed using a 4–15% Tris-glycine gradient gel run for 35 min at 200 V. After electrophoresis, the proteins were either stained with Coomassie Brilliant Blue G-250 or transferred to a nitrocellulose membrane for Western blotting (all items from Bio-Rad, Hercules, CA). A 1:200 dilution of mouse anti-BChE IgG was used as the primary antibody and a 1:2000 dilution of goat anti-mouse IgG-HRP was used as the secondary antibody (Santa Cruz Biotechnology, Dallas, TX). Immunoreactive bands were detected colorimetrically by incubation with 3,3′,5,5′-tetramethylbenzidine substrate (Promega, Madison, WI). Pure plasma-derived human BChE provided by Dr. Douglas Cerasoli at the USAMRICD was used as a control. Purity quantification was performed by image analysis using ImageJ (US National Institutes of Health, Bethesda, MD).

2.6 | Quantification of active rrBChE

Active BChE concentration in each sample was measured using a modified Ellman activity assay (Ellman, Courtney, Andres, & Featherstone, 1961). The assay monitors the hydrolysis of butyrylthiocholine (BTCh) in the presence of Ellman's reagent. This reaction was monitored using a spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA) to measure the absorbance at 405 nm over a period of 3–5 min at 25 °C and pH 7.4. Samples were diluted until the rate of the colored product formation was within 200–500 mOD/min. Each sample's concentration of total soluble protein (TSP) was determined using a Bio-Rad Protein Assay kit (Bio-Rad) according to manufacturer's instructions.

2.7 Mass spectrometry

Purified rrBChE was subjected to mass spectrometry (LC-MS/MS) based protein identification. The rrBChE was digested with trypsin and analyzed on a Thermo Scientific Q Exactive Orbitrap Mass spectrometer in conjunction with Proxeon Easy-nLC II HPLC (Thermo Scientific, Waltham, MA) and Proxeon nanospray source. The digested peptides were loaded onto a 100 μm × 25 mm Magic C18 100Å 5U reverse phase trap where they were desalted before being separated using a 75 μm × 150 mm Magic C18 200Å 3U reverse phase column. Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer (Thermo Scientific) and analyzed using X! Tandem (The GPM, thegpm.org; version CYCLONE (2013.02.01.1)) set up to search uniprot *Oryza sativa* subsp. *Japonica* (09122016, 49037 entries).

2.8 | N-terminal sequencing

The N-terminal sequence of rrBChE was determined using a Procise Protein Sequencing System (Applied Biosystems Inc., Foster City, CA), with all reagents sourced by the manufacturer. Four activity units (~5 mg) of purified rrBChE were applied to a ProSorb PVDF unit, followed by several aqueous washes and immobilization with BioBrene. Eleven cycles of automated Edman chemistry were performed on the sample using the preprogrammed pulsed-liquid PVDF method.

2.9 | Glycan analysis

Purified rrBChE was digested using sequencing grade modified trypsin (Promega) to produce peptides and glycopeptides. rrBChE was denatured in 50mM NH_4HCO_3 using 2μl of 550mM dithiothreitol (DTT) and then alkylated using 4 μl of 450 mM iodoacetamide (IAA) in the dark for 30 min. The sample was then digested using 1 μg of trypsin at 37 °C for 18 hr before it was injected into the QE-Orbitrap for LC-MS/MS analysis. The data output was analyzed to identify the peptides and glycopeptides using Byonic software (Protein Metrics, San Carlos, CA). All the glycopeptides that were observed in the tandem mass spectrometry were then quantified using the QQQ instrument.

The individual glycoforms were quantified using an Agilent 1290 infinity II UPLC system coupled to an Agilent 6495 triple quadrupole (QQQ) LC-MS (Agilent Technologies, Santa Clara, CA). An Agilent Eclipse plus C18 column (RRHD 1.8 μm, 2.1 mm × 150 mm) connected to an Agilent Eclipse plus C18 pre-column (RRHD 1.8 μm, 2.1 × 5 mm) was used for UPLC separation. A 10 min binary gradient was used at a flow rate of 0.5 ml/min,

composed of solvent A (3% acetonitrile, 0.1% formic acid in aqueous solution) and solvent B (90% acetonitrile, 0.1% formic acid). Dynamic multiple reaction monitoring (MRM) was used for the quantitation, thus reducing the number of analyte transitions monitored at any given time. The analytes were ionized in positive mode at a unit resolution. The data obtained from the QQQ were analyzed using Agilent MassHunter Quantitative Analysis version B.05.02/build 5.2.365.0software. Diagnostic glycan oxonium fragment m/z 204.08 was used for quantification after the glycoform presence was confirmed using an additional diagnostic fragment, m/z 366.14.

2.10 | Determination of inhibition rate constants for nerve agents

Quantitative comparison of the efficiency of OP nerve agent sequestration for rrBChE and hBChE was accomplished by measuring the inhibition rate constant of each enzyme for each nerve agent of interest. First, the K_M of the enzyme with BTCh is used to determine the concentration of BTCh needed in the inhibition experiment to allow the enzymatic hydrolysis to progress at maximal velocity. Next, a fixed concentration of BTCh and varying concentrations (1 pM – 1 mM) of the OP compounds tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), and methylphosphonothioic acid (VX) are added to the enzyme simultaneously. The absorbance at 412 nm is measured continuously for 5 min, and the resulting curves are fit by nonlinear regression to determine the rates of inhibition for each nerve agent concentration as previously described (Hart & O'Brien, 1972).

3 | RESULTS AND DISCUSSION

3.1 | Purification of rrBChE

In the current study, recombinant BChE was purified from extracts of rice cell culture using a combination of TFF, DEAE, and Hupresin affinity chromatography. Initially we attempted to replicate Brazzolotto et al.'s protocol by loading our crude, filtered extract directly to the Hupresin. While the purity of the sample was significantly enriched, an SDS–PAGE analysis indicated several contaminating proteins were co-eluted, and a significant amount of rrBChE was detected in the column flow through (data not shown). This may be explained by the fact that Brazzolotto et al. loaded a cell culture medium containing secreted BChE to the resin, which has a lower concentration of other non-target host cell proteins compared with a whole cell extract. As previously reported, the majority of rrBChE is found associated with the biomass rather than in the culture media, likely due to the protein's large size (Corbin et al., 2016; Huang et al., 2015).

After homogenization and clarification of the rice cell extract, a GE Life Sciences IEX Selection Kit was used to screen seven commonly used anion and cation exchange resins as potential capture steps for the process: DEAE, ANX, Q, Q XL, SP, SP XL, and CM. DEAE resin was selected as an initial capture step for its high recovery of active rrBChE, mild conditions, and pervasiveness in industrial processes.

In our preliminary process development stage, we found the binding of rrBChE to the DEAE resin to be extremely sensitive to the salt concentration in the mobile phase. Even low concentrations of salts (estimated at about 10 mM NaCl based on the conductivity increase)

derived from extraction (likely from a combination of intracellular salts released during cell lysis and media-derived salts trapped in the cell walls and aggregates) led to low binding of rrBChE to the resin. To combat this issue, TFF was used prior to DEAE chromatography to both concentrate and diafilter the extract into fresh buffer to remove extract-derived salts and other low molecular weight compounds that may interfere with rrBChE binding to the column. A 30kDa pore size membrane was chosen to ensure all rrBChE, including monomers (75 kDa), were retained in the extract. No significant change in TSP was observed during TFF, likely because a very low proportion of extract proteins are small enough to pass through the 30 kDa membrane. However, SDS–PAGE analysis shows a few bands of 25 kDa and smaller in the TFF permeate (data not shown). The TFF process provided high BChE recovery (>95%), which matches the recovery from other industrial TFF processes involving products from mammalian cell and bacterial cultures (Dominguez, Rivera, Escobar, & Weidner, 2008). The diafiltered extract had a slightly cloudy appearance that could be removed using a 0.22 pm filter. No change in active rrBChE concentration was detected after this filtration, which indicates that other host cell proteins, polysaccharides, lipids, or nucleic acids, but not rrBChE, had precipitated out of the extract due to the low salt concentration. The absence of added salt in the 20 mM phosphate buffer did not appear to affect the stability or activity of rrBChE in any way. The optimal NaCl concentration for elution is 300 mM, which eluted ~75% of the loaded active rrBChE.

Elution fractions from the DEAE resin were then combined and applied to Hupresin. The resin was equilibrated, loaded, and washed with 20 mM sodium phosphate with 150 mM NaCl at pH 7.4, eluted with 500 mM tetramethylammonium chloride (TMAC), and stripped with 500 mM NaOH after each use. Using this strategy, we consistently found ~15% of the loaded rrBChE in the column flow through and wash and 60% of the loaded rrBChE in the elution. Only ~1% of loaded rrBChE was detected in the resin stripping step, which left the remaining ~24% of active BChE unable to be accounted for. The exact cause of this is unknown, but could be due to a loss of enzymatic activity during the process, or tight binding of rrBChE to the resin that is not disrupted during elution or stripping. The Hupresin was reused for >10 purification cycles with no significant loss of binding capacity (~2 mg BChE/ml resin).

Table 1 summarizes the overall rrBChE recovery at each step of the process, the overall process yield of the sample at that step, and its percent purity. All rrBChE quantification data report only active enzyme, and use a specific activity determined by the modified Ellman assay and TSP (using the Bradford assay) of a highly pure form of rrBChE. Figure 1 shows an SDS–PAGE (Figure 1a) and Western blot (Figure 1b) under reducing conditions for the same steps described in Table 1. The banding patterns in the SDS–PAGE of the crude extract (C) and the concentrated extract after TFF (T) are similar (Figure 1a), which agrees with the minimal observed change in sample purity. Only a single major band is seen in the SDS–PAGE of the elution fractions from Hupresin (H), which corresponds to the expected size of the rrBChE monomer. The rrBChE band is more diffuse and migrates slightly further that of the hBChE control, which is likely explained by rrBChE's plant glycan structures. Western blotting confirms the presence and relative concentration of rrBChE in each of these samples. Image analysis of the SDS–PAGE indicates a >95% purity of the rrBChE eluted from Hupresin.

3.2 | rrBChE kinetic parameters

The kinetic behavior of rrBChE and hBChE were studied by performing an Ellman assay on the molecule at varying concentrations (0–5 mM) of BTCh and using a nonlinear regression to fit the data to a kinetic model developed for BChE (Radic, Pickering, Vellom, Camp, & Taylor, 1993) (Figure 2a). The data from the nonlinear regression was then used to determine several kinetic parameters (Figure 2b). For all calculated parameters, the recombinant and native forms of the molecule had comparable values, with no statistically significant differences. Furthermore, the kinetic parameters for hBChE are comparable to what has been reported in other studies (Alkanaimsh et al., 2016; Geyer et al., 2010; Radic et al., 1993).

3.3 | rrBChE activity as a function of pH and temperature

Ellman assays were also performed at varying pH (Figure 3a) and temperature (Figure 3b) to determine the relationship between the enzyme's activity and those two factors. The studies on pH were performed by preparing both assay substrates and sample dilutions in 100 mM sodium phosphate buffer at pH 5, 6, 7, 7.4, and 8, and subsequent calculations utilized pH-specific absorption coefficients (Masson et al., 2003). Both enzymes show very low (but non-zero) activity at pH 5, with a gradual rise in activity to pH 8. The two curves follow a similar trend, with comparable relative changes in activity and an inflection point in the curve at a pH between 6 and 7.

The temperature studies were performed by storing aliquots of rrBChE and hBChE at 4, 22, 30, 40, 50, 60, 70, and 95 °C for 30 min before assaying them in standard conditions at 25 °C (Figure 3b). The data confirm that both rrBChE and hBChE maintain their activity over long periods when stored at 4°C (Lockridge et al., 2005), and for shorter periods when stored between 4 and 40 °C. Above 60 °C, all activity is lost from both molecules within 30 min. However, we consistently found that hBChE incubated at 50 °Cfor 30 min exhibited higher activity than when it was incubated at any other temperature. This same trend was seen for native equine BChE (data not shown), but not for rrBChE. In this temperature range, hBChE has been found to follow complex, non-linear thermal inactivation kinetics. In particular, increased stability and altered catalytic properties could explain the apparent increase in activity that we see in this study (Cengiz, Cokuğraş, & Tezcan, 2002). However, it is not obvious why rrBChE does not share this behavior.

3.4 | rrBChE amino acid sequence determination

To confirm the amino acid sequence of rrBChE, the molecule was subjected to LC-MS/MS analysis. Figure 4a shows the aligned sequences of hBChE and rrBChE, with N-glycosylation sites bolded, and portions thatwere not clearly detected by LC-MS/MS represented with dashes. We observed a peptide coverage of 86% and 188 unique peptides, with undetected sequences containing a high density of trypsin cleavage sites, making it difficult to identify the residues in that region. We also performed N-terminal (Edman) sequencing on rrBChE (Figure 4b). All eleven of the identified residues match the N-terminal sequence of hBChE, which confirms that there is no N-terminal cleavage of the recombinant molecule and that the RAmy3D secretion signal peptide is properly cleaved.

Based on these analyses and the activity data, the purified rrBChE likely consists of the full, mature BChE sequence.

3.5 | Abundance and structures of N-glycans

The site-specific N-glycosylation pattern of rrBChE (Figure 5) was mapped using tandem mass spectrometry and then quantified using triple quadrupole (QQQ). The numerical representation of glycan structure represents the number of hexoses, HexNAc, fucose, and xylose, respectively. For example, the GlcNAc(Xyl)Man3(Fuc)GlcNAc2 (3311) glycoform contains three hexoses (i.e., mannose), three GlcNAc's, one fucose and one xylose residue.

BChE contains 10 resides that could potentially be modified with N-glycans (N17, N57, N106, N241, N256, N341, N455, N481, N485, and N486). Figure 5 shows that at least six sites were occupied with glycans. Sites N481, N485, N486 are contained within the same tryptic peptide, so the glycoform identified on that peptide could not be assigned to any individual site within that peptide sequence. No glycopeptides were found that match sites N57 and N106, though this does not necessarily indicate that these sites are aglycosylated.

Overall, the N-glycans structures found on rrBChE match typical, complex biantennary plant N-glycan structures that would be expected for a product secreted from a plant cell. Most of the detected structures contained an α-1,3 linked core fucose and a β-1,2 linked xylose (Figure 5b). The presence of these structures suggests that, despite low detection of rrBChE in the culture medium, the molecule is indeed being processed through the secretory pathway as intended. The structure, relative abundance, and heterogeneity of these glycans across the sites match what has been previously reported for recombinant proteins secreted from rice cell suspension cultures (Jung, Kim, Jang, Shin, & Yang, 2016; Kang et al., 2015), and are similar to those secreted from whole *Nicotiana benthamiana* plants (Alkanaimsh et al., 2016; Schneider, Marillonnet et al., 2014). Glycans terminating in galactose residues are likely a result of incomplete formation of the LewisA structure, which is commonly found in the rice secretome (Jung et al., 2016), though not always detected in high abundance (Kang et al., 2015; Shin, Chong, Yang, & Kwon, 2011).

While the structures themselves differ, the site-specific glycan heterogeneity on rrBChE correlates with some of what is seen in hBChE. Sites N256 and N341 are most heterogeneous for rrBChE, with 7 and 10 different structures detected at each site, respectively. The corresponding sites on hBChE have 13 and 8 different structures. However, for all other sites detected for rrBChE, the recombinant molecule had far fewer different glycan structures (1–5) than on the corresponding sites for hBChE (8–13) (Kolarich et al., 2008). This indicates that rrBChE is a more homogeneous form of the molecule, which is a desirable trait for therapeutic molecules. This is also helpful for future work on glycan humanization (removal of plant-specific residues and the addition of terminal sialic acid), which we expect would enhance the pharmacokinetic stability and reduce the risk of immunogenicity (Saxena et al., 1998). Such glycan modification can easily be performed in rice cell suspension culture through either glycoengineering of the host cells (Shin et al., 2011; Sim, Lee, Chung, Kim, & Hahn, 2015) or post-purification, in vitro glycan modification.

3.6 | Organophosphate inhibition rate constants

The most compelling indicator of rrBChE's likely in vivo efficacy is its ability to sequester OP compounds in vitro (Ashani et al., 1991). To assess this, we measured the inhibition rate constants of five OPs for both rrBChE and hBChE and compared them (Figure 6). Although the inhibition rate constants of rrBChE for all OPs are different from the values for hBChE (unpaired *t*-test, $p = 0.01$), all measured values exceed the empirically determined minimum value needed for a stoichiometric bioscavenger to provide protection against OP intoxication (10^6 M^{-1} min^{-1}). Given these results, rrBChE will likely prove to be efficacious in vivo against all tested OPs. Other studies of recombinant BChE from different expression systems have cited a reduced affinity for inhibitors when compared to hBChE (Geyer et al., 2010), while others have assessed the inhibition but not compared it to hBChE (Huang et al., 2007).

Plant-specific glycan structures could, in part, explain the observed differences in OP binding between rrBChE and hBChE. While previous studies of hBChE have indicated that removal of entire glycans can leave catalytic activity unaffected (Nachon et al., 2002), which is supported by the fact that no glycans are located near the entry to the active site (Fang, Zheng, & Zhan, 2014), reports describing the ability of hBChE with altered glycan structures to bind OPs are limited. Because this in vitro measurement is being used as an initial evaluation of the potential efficacy against OP toxicity that could be provided by rrBChE, the actual value of the inhibition rate constant is not as important as the fact that the value is higher than 10^6 M^{-1} min^{-1}, indicating that it will likely provide protection against exposure to all tested OPs.

4 | CONCLUSIONS AND FUTURE PROSPECTS

Recombinant BChE extracted from transgenic rice cell culture was purified using TFF, DEAE chromatography, and Hupresin chromatography to give an overall process yield of 42%. The recombinant molecule was found to be functionally and structurally similar to native hBChE, except for the structure of its N-glycans, which are made up of typical complex, biantennary plant glycan structures. Calculation of the inhibition rate constants for rrBChE and various OPs indicate that rrBChE will likely be an effective bioscavanger against these molecules in vivo.

Future work will include further process development to characterize and improve process yields, including optimization of extraction, clarification, and chromatography protocols, with special attention to developing a process that is amenable to current industry practices. We are also working on in vitro enzymatic glycan modifications for potential circulatory half life enhancement. In vivo studies will confirm the molecule's efficacy as a bioscavenger and allow pharmacokinetic analysis. A technoeconomic analysis for rrBChE production from transgenic rice cell cultures is also needed to assess the economics of the entire production and purification process.

ACKNOWLEDGMENTS

Funding for this project was provided by the National Institutes of Health, National Institute of General Medical Sciences (NIGMS-NIH, #T32-GM008799) and the Defense Threat Reduction Agency (DTRA,

#HDTRA1-15-0054). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIGMS, NIH, or DTRA. We would like to dedicate this article to one of our coauthors and eminent scientist of USAMRICD, Dr. Douglas M. Cerasoli, who was the major force in this interdisciplinary work and known for his dedication for chemical defense research.

Funding information

Defense Threat Reduction Agency, Grant number: #HDTRA1-15-0054; National Institute of General Medical Sciences, NIH, Grantnumber: #T32-GM008799

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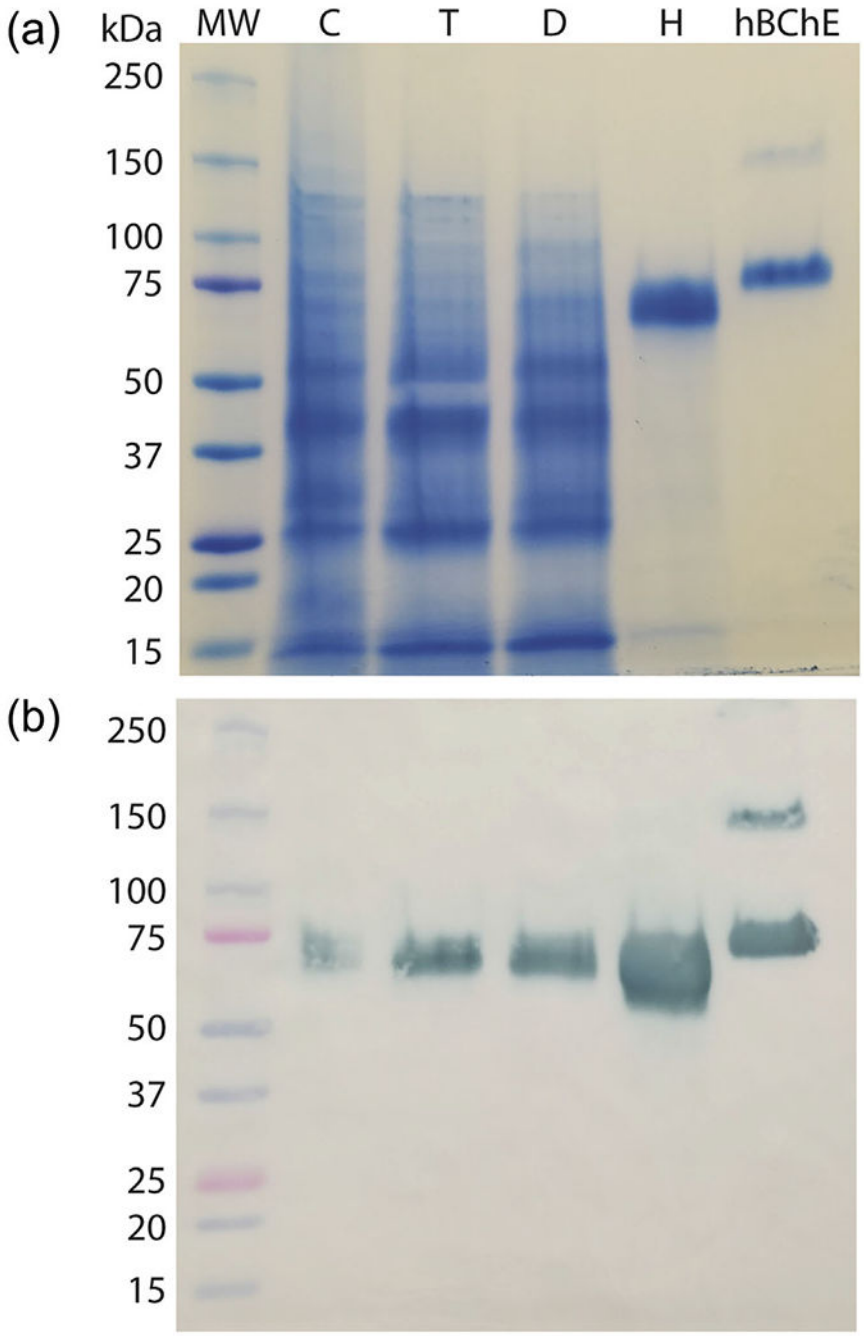
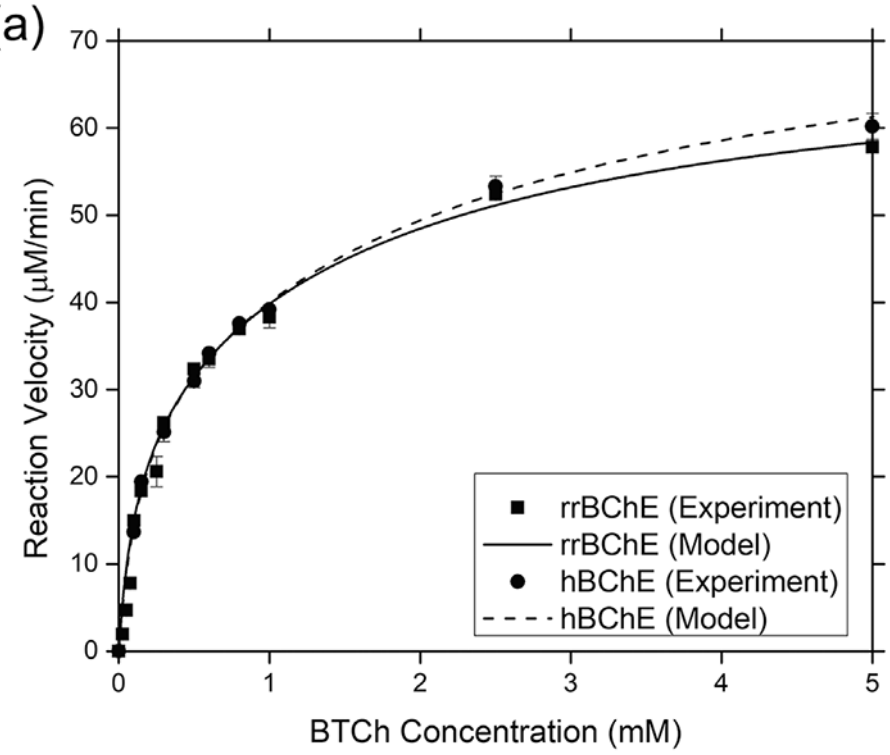


FIGURE 1.

SDS–PAGE (a) and Western blot (b) of purification steps of rrBChE. MW, molecular weight protein standards; C, 30 μl crude cell extract (~70 ng rrBChE); T, 4 μl concentrated extract from TFF (~400 ng rrBChE); D, 4 μl combined elution fractions from DEAE chromatography (~560 ng rrBChE); H, 12 μl combined elution fraction from Hupresin chromatography (~4.5 μg rrBChE); hBChE, 5 μg purified human BChE

(a)



(b)

	rrBChE	hBChE
K_m (μM)	112 ± 55	135 ± 42
K_{ss} (mM)	1.8 ± 1.0	2.4 ± 0.9
b	2.4 ± 0.6	2.4 ± 0.3
k_{cat} (10^4 min^{-1})	12.4 ± 4.6	15.0 ± 3.4
k_{cat}/K_m (10^8 min^{-1} M^{-1})	11.0 ± 6.7	11.1 ± 4.3

FIGURE 2.

Kinetic analysis of rrBChE. The rate of hydrolysis of varying concentrations (0–7.5 mM) of BTCh by rrBChE (a) was monitored over 5 min in 100 mM sodium phosphate buffer with 0.267 mM DTNB at pH 7.4. A nonlinear regression was performed to calculate kinetic parameters for rrBChE (b)

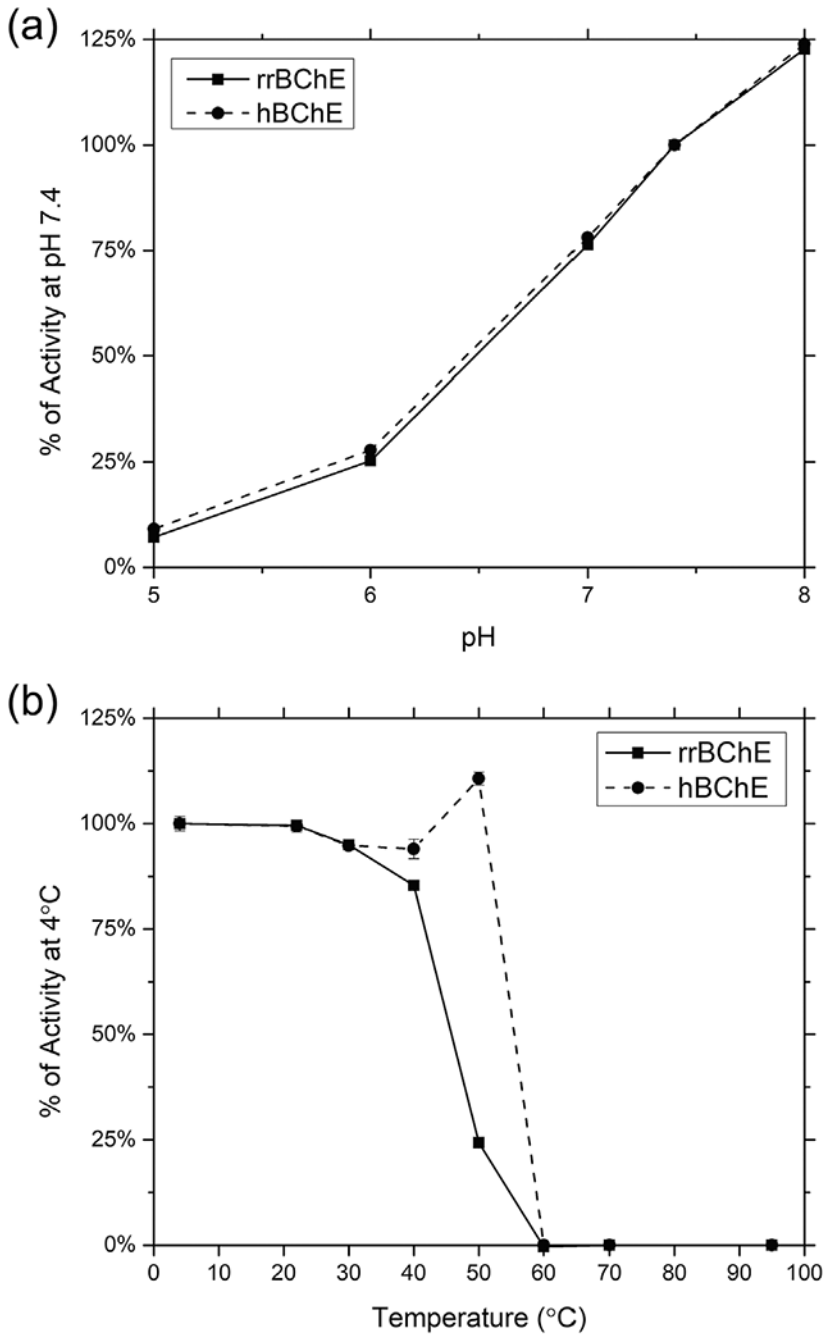
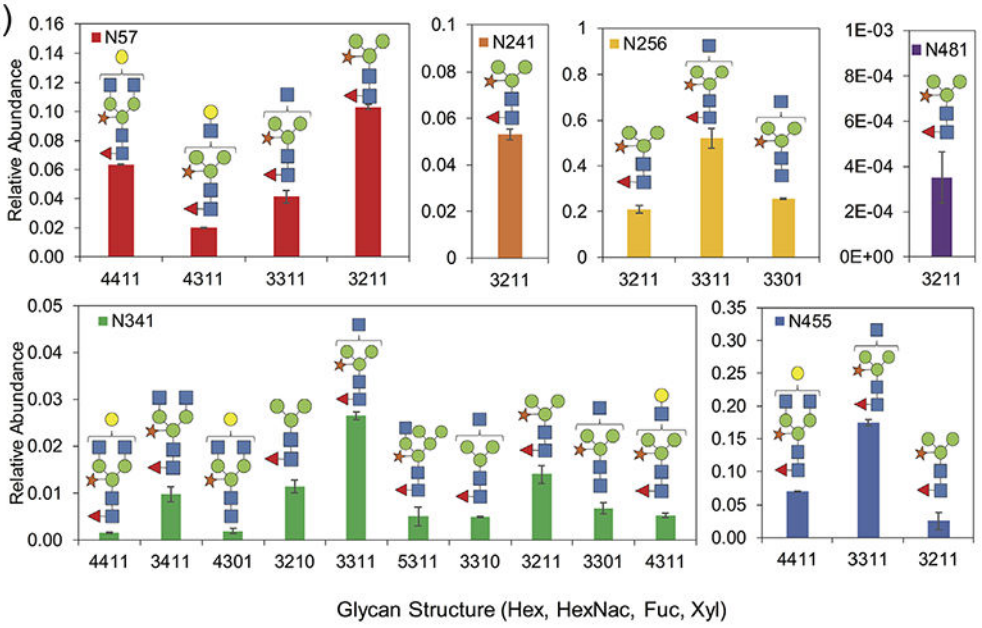


FIGURE 3.
rrBChE activity as a factor of (a) pH during activity assay and (b) temperature during 30 min incubation

FIGURE 4.

rrBChE sequence as determined by (a) LC-MS/MS and (b) N-terminal sequencing. Residues that were not identified are represented with a dash (–) and N-glycosylation sites are bolded

(a)



(b)

Glycan Structure	N57	N241	N256	N341	N455	N481
MMF	<1		<1	13		
MMXF	45	100	20	16	9	100
GnMX			25	8		
GnMF				6		
GnMXF	18		51	30	64	
GnGnX				1		
GnGnXF			<1	11		
AGnXF	28		2	2	26	
AMX				2		
AMXF	9		<1	6	2	
% Containing Fucose:	100	100	74	88	100	100
% Containing Xylose:	100	100	100	80	100	100

FIGURE 5.

Site-specific and overall N-glycan structures on rrBChE. (a) The relative abundance of glycopeptide MS signals are grouped by N-glycosylation site into each graph. Sites N481, N485, and N486 are all contained in the same tryptic peptide and are grouped together under the label for N481. (b) The percent relative abundance of each structure at each specific site is shown, based on ratios of signal intensities from glycopeptides matching that residue. Due to rounding to whole numbers and removal of extremely low abundance structures, the sum of these may not add to 100

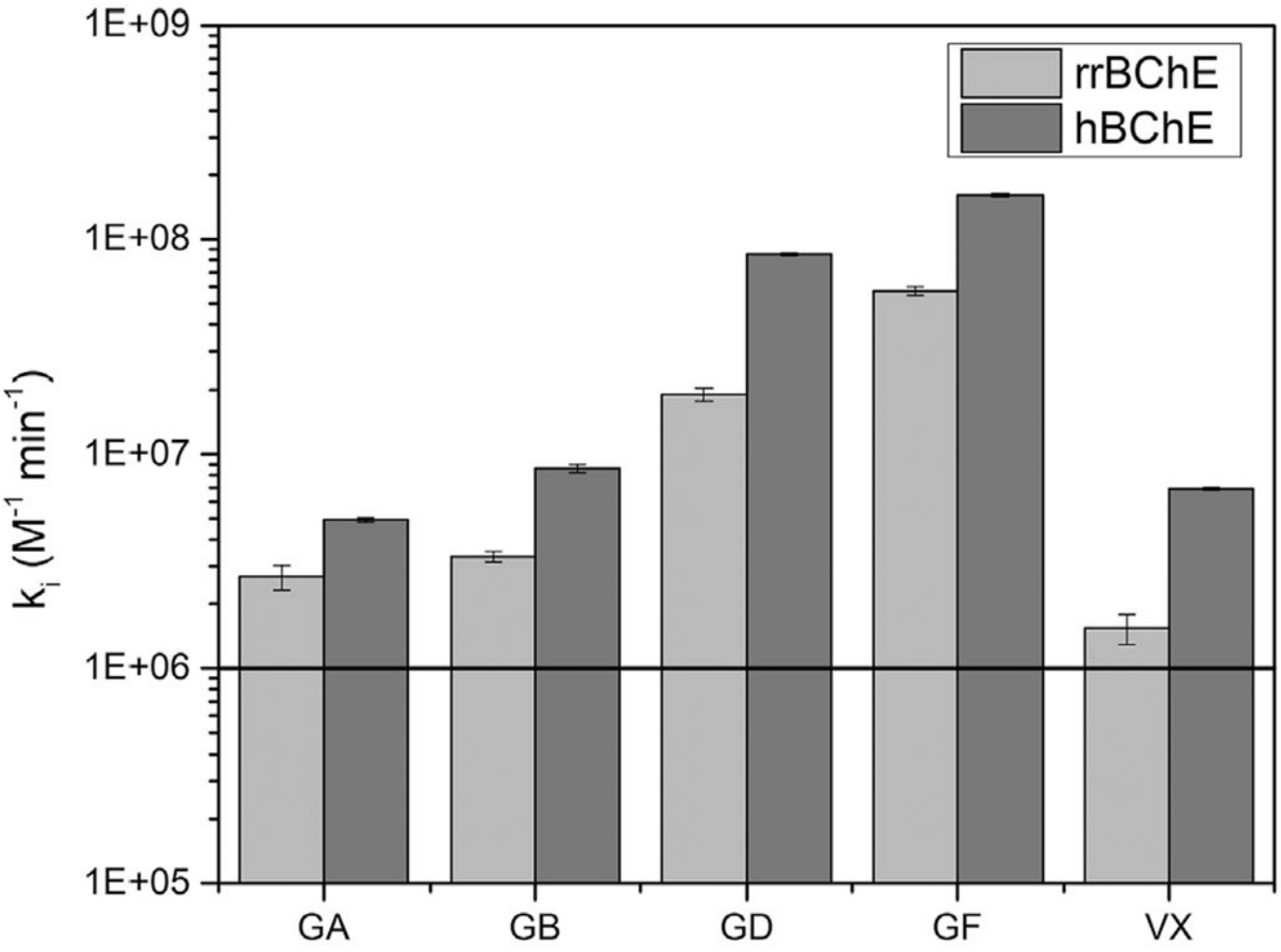


FIGURE 6.

Inhibition rate constants for rrBChE and hBChE for various organophosphate compounds. The estimated minimum inhibition rate constant of a stoichiometric scavenger capable of providing protection against intoxication (10^6 M^{-1} min^{-1}) is indicated by the solid black line. Organophosphates studied are tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), and methylphosphonothioic acid (VX)

TABLE 1

Recovery of active rrBChE from purification steps

Step	% Yield (step)	% Yield (overall)	% Purity (mg rrBChE/mg TSP)
Crude extract	100	100	0.9
TFF	95	95	0.9
DEAE	75	70	1.8
Hupresin	60	42	>95