



Advancements in recombinant technology for production of butyrylcholinesterase, a bioscavenger of nerve agents

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Abbreviations:

AChE – acetylcholinesterase
AAV – adeno-associated virus
BChE – butyrylcholinesterase
hBChE – human butyrylcholinesterase
rBChE – recombinant butyrylcholinesterase
HUCPVCs – human umbilical cord perivascular cells
EF-P – elongation factor protein
NA – nerve agents
OP – organophosphorus compound
PRAD – proline-rich attachment domain
PROSS – protein repair one stop shop
WAT – tryptophan amphiphilic tetramerization

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Abstract

Butyrylcholinesterase (BChE) is a serine hydrolase present in plasma and other mammalian tissues. As a target of organophosphorus pesticides and warfare nerve agents, BChE acts as their stoichiometric bioscavenger. However, so far it has been a significant challenge to produce BChE at large scales and low cost. For decades, numerous research efforts have been directed first at isolation from human volunteers and later at production of BChE in eukaryotic and prokaryotic expression systems. In this review we focused on recent studies on recombinant BChE discussing reasons why the efficient, economically sensible expression system for recombinant BChE is hard to develop. We also bring the most recent advancements in the use of expression of human BChE *in vivo* as an effective prophylactic against organophosphate poisoning.

INTRODUCTION

The widespread use of organophosphorus compounds (OPs), primarily pesticides, but also the availability of highly toxic nerve agents (NA), generates a significant number of poisonings worldwide; leading up to several hundred thousands of deaths per year (1). From the moment of their synthesis, organophosphorus compounds have become a constant threat throughout history and have maintained their presence to this day. Taking that into consideration, countermeasures for OP poisoning should pursue the same pattern or even be one step ahead. Standardized care in cases of NA poisoning with sarin (GB), soman (GD), tabun (GA) and VX is based on a combination of anti-muscarinic antagonist atropine, reactivators of NA-inhibited acetylcholinesterase (AChE, EC 3.1.1.7), and an anticonvulsant (2). Although conventional nerve agent countermeasures have effects on survival rates depending on the time of application, shortcomings in the prevention of central nervous system exposure are commonly observed as convulsions or brain damage (3). Pre-treatment with bioscavengers, enzymes that rapidly bind OPs and reduce their free levels in circulation can prevent long term health effects in the central nervous system imposed by OP-inhibition of the synaptic AChE. For enzymes to be used as bioscavengers they should act rapidly and against a broad spectrum of NAs, have prolonged circulation time (ideally more than 10 days), have no immunogenic or toxic properties, be available at sufficient concentration and at reasonable cost (4–6).

The most investigated bioscavenger is human plasma butyrylcholinesterase (BChE, EC 3.1.1.8), serine hydrolase and enzyme analogue of AChE. Although its physiological function is not essential as that of AChE, it seems that it plays a back-up role in maintaining and regulating the cholinergic activity (7). Also it has been shown that BChE is involved in metabolism of drugs including cocaine, heroin and aspirin (8–10) as well as in physiological pathways of ghrelin, the appetite-promoting hormone which indicate its possible role in lipid metabolism (11, 12).

Given the pharmaceutical importance of BChE, the goal is to find a way to produce BChE in large quantities. Human population has a wide range of plasma BChE concentration from 3.5 to 9.3 mg/L (7, 13). Isolation of hBChE from obsolete plasma or Cohn fraction IV-4 is possible on a laboratory and industrial scale using ion exchange chromatography at pH 4 and affinity chromatography on procainamide-Sepharose or Hupresin to obtain purified plasma hBChE from 100 L in single cycle (14, 15). Outdated human plasma is a reliable source for the hBChE tetramer which is important for long half-life and bioscavenging activity, but production is expensive and time-consuming, since isolation requires large amounts of human plasma to achieve low levels of purified hBChE (13). An alternative method for obtaining large amounts of BChE is via synthesis of recombinant proteins in different expression systems. However, hBChE has proven difficult to reproduce by recombinant technology because it's hard to accomplish fully active tetrameric form. Problem with most of recombinant expression systems is based on inadequate glycosylation, and deficient oligomerization of expressed BChE. In the majority of expression systems recombinant BChE are found as monomers or dimers which lead to low half-time in circulation and unsatisfactory pharmacologic properties (16,

17). Considerable effort has been embedded into developing transgenic recombinant platforms to synthesize fully functional recombinant hBChE cost-effectively and on a large scale, and this task is still under way.

THE IMPORTANCE OF STRUCTURE FOR FUNCTIONAL BChE

hBChE is a glycoprotein formed from four identical subunits encoded by gene localized on chromosome 3 (3q26) (18). Each monomeric subunit has a molecular weight of 85 kDa consisting of 574 amino acid residues, 24% of weight is consistent with 9 polysaccharide chains *N*-linked with asparagine residues of protein. hBChE is present in several oligomeric forms, of which the tetrameric form in the serum is most abundant, and the remaining is represented by trimeric, dimeric and monomeric forms (7). Each monomeric hBChE subunit contains a catalytic triad (Ser198, His438, and Glu325) within a 20 Å deep active site gorge. Two domains differ in native hBChE structure, the core or catalytic domain (amino acids 1–529) and the tetramerization domain (amino acids 530–574). The tetramerization domain of hBChE is formed by C-terminal tryptophan amphiphilic tetramerization (WAT) helices from each subunit as a superhelical assembly around a central polyproline (19). The WAT domain can connect with the proline-rich attachment domain (PRAD) sequence of ColQ or PRIMA protein anchors that enable cohesion of BChE to the synaptic membranes of nervous system (20). Polyproline-rich peptides associated with BChE are mostly derived from lamelliopodin (~70%), membrane-associated protein (21, 22). PRAD sequence in WAT/PRAD complex adopts a polyproline II helical conformation and runs antiparallel. This complex is important to mediate the congregation of

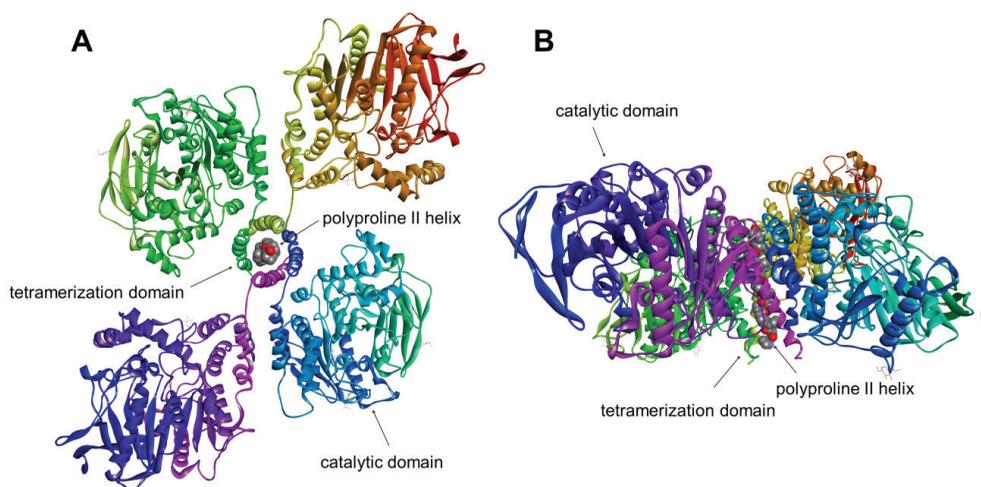


Figure 1. Cryo-EM structure of the native BChE tetramer (PDB 6I2T). A) Top view of the BChE tetramer as dimer of dimers in which the monomers are diagonally equivalent with polyproline II helix in the centre. B) Side view of the BChE tetramer with tetramerization domain concealed between dimers.

tetrameric native hBChE which displays effective therapeutic action in the presence of OP compounds or other drugs like cocaine (19, 23–26).

The crystal structure for recombinant hBChE monomer (PDB 1P0I) was solved with truncation at C-terminus of 40 amino acid residues in the tetramerization domain (27). Crystal structure of full-length glycosylated tetrameric hBChE by X-ray crystallography has not been determined so far, but alternative method emerged. Cryogenic electron microscopy provided 3D structure of hBChE (Figure 1) using crystal structure for recombinant hBChE with all the N-glycosylated sites presents (PDB 4AQD) and WAT helices based on the synthetic WAT/PRAD complex of AChE (PDB 1VZJ) (28, 29). The cryo-EM structure of tetramer hBChE (PDB 6I2T; EMD-0256) is a dimer of dimers stabilized by a superhelical assembly which could be the key to future advancements in recombinant technology for BChE production. The obtained model showed catalytic domains within dimers asymmetrically linked to the WAT/PRAD complex inducing concealment of the tetramerization domain between catalytic units which can contribute to the stability of the tetrameric form of hBChE (30, 31).

An important role in the stabilization of the tetrameric structure can also be the contribution of a high glycosylation level of BChE which in past studies was assumed as an impairment to get proper hamper of crystals but a model obtained with a combination of cryo-EM, small-angle X-ray scattering and molecular dynamics simulations allowed us new insights. The acquired model clearly shows that some glycans interact between monomers of bordering dimers. These glycans are linked to asparagine 241 of one monomer and asparagine 256 of the adjoin monomer thus strengthening the tetramer stability in addition to the C-terminal knot (31). Post-translation modification as *N*-glycosylation except for tetramer stability of BChE can also contribute to immunocompatibility, reactivity and pharmacokinetic stability of enzyme in circulation (32). Except for glycosylation for obtaining longer half-life in circulation, high importance is given to inter monomeric disulfide bonds and sialylation for prevention of binding BChE-glycans to the asialoglycoprotein receptor in liver (33, 34).

Accumulation of BChE in aggregates was promoted by organophosphorus pesticide, chlorpyrifos oxon ethyl, that induces crosslinking by isopeptide bond leading to dimer, trimer, and higher complexes of BChE (35). The most well-defined isopeptide crosslink between monomers was lysine 544 to glutamic acid 542 located in the C-terminus tetramerization domain. It is worthy to mention that the protein aggregates are a characteristic feature of neurodegenerative diseases such as Parkinson's and Alzheimer's, and OP-induced crosslinking of proteins might be a link between pesticide exposure and the development of some cases of Alzheimer's and Parkinson's diseases (35).

HUMAN BCHE AS A BIOSCAVENGER OF NERVE AGENTS

hBChE was selected as the most promising bioscavenger candidate for further development in 2007 when it acquired research status for a new drug from the US Food and Drug Administration. Bioscavengers as, in this case, OP-reacting proteins, must meet certain standards which account for no behavioural and physiological side effects and also protection higher than 5 LD₅₀ of more than one nerve agent. hBChE rapidly binds nerve agents and having high half-time in circulation of 12–15 days can provide extended protection of the synaptic AChE against nerve agents while avoiding unwanted immunological responses (2, 4). BChE can be defined as a stoichiometric bioscavenger, meaning that it has covalent interactions with OP compounds in a 1:1 ratio, *i.e.* one molecule of BChE can only remove one molecule of OP in blood. In this way, the concentration of the OP compound in the bloodstream is lowered, but the enzyme remains practically permanently inhibited. Therefore, although stoichiometric bioscavengers are very effective, they must be applied at a high concentration for the OP compound to be removed within a single circulation period (4). A hBChE dose of 2400 nM which corresponds to 200 mg per 70 kg is a prophylactic in case of human exposure to a dose of 2–5 LD₅₀ nerve agents (5, 36). Animal studies have shown that administration of a higher dose of hBChE would provide protection from exposure to 5.5 LD₅₀ GD and 8 LD₅₀ VX (26). Higher doses can easily trigger an immune response but testing on mice with a 800-times higher dose than in their system has shown no unwanted effects on health. Moreover, intravenously or intramuscularly applied plasma-derived hBChE was regarded as safe in phase I clinical trials (4, 13).

The use of hBChE as a stoichiometric scavenger requires a high concentration of named enzyme which is hard to come by, so the feasibility of other approaches was explored. Catalytic systems combined of bioscavengers as BChE and reactivators as oximes seemed like a possible solution considering that they can neutralize substantial amounts of OP molecules by cycles of inhibition and reactivation, making requirements for BChE concentrations lower. This pseudo-catalytic system is limited by the effectiveness of reactivators to induce dephosphorylation of the enzyme active serine. Unfortunately, standard reactivators such as 2-PAM, HI-6 and obidoxime are mainly intended for the reactivation of AChE and the reactivation of BChE-OP conjugates by these compounds is inefficient (37). In the last decade, interest in the design of BChE reactivation-specific oximes has emerged so groups like quaternary benzaloximes (38), imidazolium and benzimidazolium oximes (39), chlorinated pyridinium oximes (40) are appearing and opening ground for future research in pseudo-catalytic BChE-based systems, which remains relevant.

EXPRESSION OF RECOMBINANT BChE IN PROKARYOTIC SYSTEMS

Prokaryotic cells in terms of technology are the simplest and most economically acceptable system for producing recombinant proteins. Nevertheless, attempts to express native BChE in *Escherichia coli* have so far been unsuccessful. One of the reasons was presence of three inter monomeric disulfide bonds unable to form in *E. coli* causing accumulation of partially folded BChE as inclusion bodies (7). Ongoing problem with this expression system is also related to the prokaryotic inability of post-translational modifications of mammalian proteins *i.e.*, *E. coli* does not have its own glycosylation system. *Campylobacter jejuni* has a developed *N*-glycosylation system due to the enzymatic cascade of alkaline polygalacturonate lyase, and glycosyltransferase. The transfer of this system to the genetically known bacterium *E. coli* would allow production of recombinant glycoproteins that makes 2/3 of eukaryotic proteins. However, for the time being, this mechanism can only be used for glycosylation of *C. jejuni* proteins because bacterial *N*-glycans are completely distinct from any known eukaryotic glycan. Challenges facing this post modification can possibly be overthrown by the availability of needed glycan precursors by design gene knockout strains that overproduce glycan precursors (41–43).

As stated before, glycosylated BChE is interesting from a pharmacological point of view as a tetramer, because of its longer retention capacity and its better pharmacokinetic profile than a dimer or monomer. Tetrameric BChE in prokaryotes is difficult to obtain also due to the lack of proline-rich peptides that, interacting with the C-terminal BChE domain, promote oligomerization. The proline-rich sequences in prokaryotic expression are omitted since such amino acid sequence slow down the translation of natural and modified gene expression. The polyproline mRNA motif causes the ribosome to slow down, thereby reducing the efficiency of translocation, the role of which, although not fully elucidated, is associated with gaining time for co-translational formation and membrane insertion. To reduce ribosome retention, prokaryotic cells use an EF-P (elongation factor protein) that enters the empty E site into the ribosome and binds close to the peptidyl-tRNA. EF-P contains a lysine residue modified by the enzymes YjeK and YjeA allowing the β -lysyl moiety on Lys34 reach the active site of a ribosome and restore catalytic activity. EF-P is found in less than 100 *E. coli* proteins, which describes a small number of enzymes containing polyproline motifs in prokaryotes (44–46). For the introduction of polyproline in the prokaryotic expression system, it is necessary to develop a system for the co-expression of EF-P or use an *E. coli* species with an already existing peptide (47).

Recently, Goldenzweig *et al.* (48) reported a computational method based on the structure and sequence for

the design of stable proteins for prokaryotic expression, Protein Repair One Stop Shop (PROSS). After successful expression of human AChE in the bacterial system, the same possibility arose for BChE. The PROSS system uses two filters that consider each mutation individually, limiting the design of the protein to mutations that could further contribute to the stability of the structure. Excluding context-dependent mutations leads to the minimization of false positives risk, *i.e.* structures that are stable *in silico* but unstable *in vivo* (48). The PROSS system proposed 7 variants of BChE that were expressed in *E. coli* strain as a fusion protein of thioredoxin with preserved amino acid residues of the catalytic triad and gorge of the active site to allow safe substrate binding. Surface residue optimization and protein stabilization suggested by the PROSS algorithm allowed isolation of active BChE, which was then purified to homogeneity. The purified rBChE of prokaryotic expression contains 47 intentional mutations with intact active gorge granting it similar kinetic activity as of rBChE produced in chinese hamster ovaries (CHO) cells or plasma derived hBChE (49, 50). As post-translational *N*-glycosylation and presence of polyproline peptides were excluded from prokaryotic BChE expression, it was essential to preserve amino acid residues during PROSS analysis that maintain the homodimeric structure of the protein. The isolated protein according to SEC-MAL analysis behaves as a monomer (70 kDa) despite the conservation of residues included in the dimer interface. The presence of two mutations in the immediate vicinity of the last helix, Gln518His and Thr523Asn, are the suspected reason for impairment of dimer formation. However, the crystal structure reveals a dimeric form surely driven by a higher local protein concentration (51). The homodimer is connected by a bundle of four helices, each monomer joined by a single pair of spirals forming canonical dimer observed before (28). The main difference in structure between human BChE expressed in *E. coli* and in eukaryotic expression system is recognized as a Cys65-Cys92 disulfide bond. It has been established based on the amino acid residue occupancy that there has been a partial breakdown of the linkage between the chains (51). An additional E377C/A516C mutation to the prior BChE mutant provided a cross-subunit disulfide bond featuring a full dimeric form with unaffected catalytic activity in comparison to BChE without the additional mutation. BChE with 48 mutations has shown improved thermostability justifying that disulfide bonds are important for dimerization and stability of BChE (52).

Previously it was not possible to express proteins that contain disulfide bonds in prokaryotes because of an unfavorable reduction environment in bacterial cytoplasm (53). *E. coli* SHuffle® strains based on *trxB* suppression have enhanced capacity to correctly fold proteins with multiple disulfide bonds in the cytoplasm. SHuffle® strains also contain constitutively expressed, cytoplasmic,

disulfide bond isomerase to aid in the formation of disulfide bonds and promote proper protein folding (54, 55). The ability to form wanted inter monomeric disulfide bonds impedes misfolding and generation of unfunctional proteins into the inclusion body. This is evident from two disulfide bonds fully formed Cys252-Cys263; Cys400-Cys519 in prokaryotic BChE without palpable denaturation and no major differences in structure of recombinant BChE. It is important to emphasize that by optimizing surface residues and stabilizing proteins, it was possible to isolate and purify homogeneous, active butyrylcholinesterase thanks to the PROSS algorithm (51, 56). Further research to improve glycosylation process and additional expression of the polyproline peptide prokaryotic expression system could be the answer for the production of the therapeutic butyrylcholinesterase protein.

EXPRESSION OF RECOMBINANT BChE IN EUKARYOTIC SYSTEMS

Expression of rBChE in eukaryotic cells has been yielded with reliable activity in different mammalian cells as CHO, HEK293, COS-7 counting production up to 5 mg/L. Technical means such as spinner, roller bottles or bioreactors help with the higher production of wanted enzymes, which for BChE should be at least 50–100 mg/L to hold as an affordable expression system (5, 13, 16). Large scale expression of BChE is often followed by an increase of protein misfolding due to the overload of post-translation modification in eukaryotic cells leading to production of a certain amount of inactive protein. The development of a system with adequate co-expression of peptides or enzymes included in post-translation adjustments can help avert decreases in specific BChE activity. The co-expression of the PRAD peptide during BChE expression in the eukaryotic system or the addition of chemically synthesized polyproline to the growth medium can increase tetramer production by 70% (23, 57). Transfection of expression vectors containing EF-1 promoter with proline-rich chaperons in CHO cells can increase production of active rBChE to 40 mg/L (16, 58). BChE expressed in CHO cells showed longer retention times but still not as long as plasma-derived BChE (57–59). Regardless of improvements, mammalian cells as an expression system of BChE are still economically undesirable.

Recombinant BChE was also successfully expressed in the silkworm (60) *Nicotiana benthamiana* (61), rice (62), and insect cells (28) with proper enzymatic activity but poor yield. In cases of enzymes from silkworms and transgene plants, incomplete or improper glycosylation occurs which can cause instability of rBChE. Even though there is a possibility to isolate tetrameric plant-derived rBChE (63), nonhuman glycan structure presents the issue of being recognized as an immunogen. Also, there is need of co-expression for sialylation which has a significant role

in the pharmacokinetic behavior of a BChE *in vivo* (34, 64). An industrial production of rBChE from milk of transgenic goats allows a high amount of fully active rBChE (up to 5 g/L) that is low-glycosylated mostly monomers or dimers. It has been shown that the PEGylation of rBChE prolonged its half-life in blood stream to 40–45 hours in pigs (65). Whereas rBChE derived from milk of transgenic mice had better pharmacokinetic properties even without PEGylation ($t_{1/2}$ = 32 h in pigs), the production yield was low (in µg/L) (66). Except for PEGylation as a tool to improve circulation half-life of monomers or dimers expressed in milk but also in silkworms, transgenic plants, *etc.*, the solution could be nanocapsules. Encapsulating BChE in zwitterionic polymer gel layer can protect it from denaturation and improve pharmacokinetic properties of rBChE (67). For now, PEGylated BChE from the milk of transgenic goat is implemented as prophylaxis or treatment in case of OP use until a better source emerges (65).

A novel source of therapeutic BChE was recently investigated where a fully functioning tetrameric enzyme was produced without the need for modifications by other systems. Mesenchymal stromal cells isolated from Wharton's Jelly of umbilical cord known as human umbilical cord perivascular cells (HUCPVCs) are the key for this therapeutic platform (68). This expression system is suitable for producing native BChE but not in high amounts. The paradigm that HUCPVCs has cellular machinery that protects and synthesizes genetic material of BChE with continuous emission in circulation can be used to increase enzyme output with transgene encoding of hBChE controlled by a proper promoter. To take full use of this system for BChE expression there is need to disable the internal regulatory system of HUCPVCs that inhibit accumulation of BChE in the extracellular matrix (68). The expression system provided by HUCPVCs could be a future answer for *in vivo* delivery of BChE, minimizing the necessity for reiterative dosing.

VIRUS-MEDIATED EXPRESSION OF BChE

The *in vivo* delivery system was previously investigated as a persistent source of stoichiometric bioscavenger BChE to uphold sufficient levels essential for OP neutralization. Direct delivery to system would surely reduce the cost and protein loss with bypassing enzyme purification. Adenovirus-mediated delivery of hBChE to organs as liver or muscle that are capable to express functional hBChE has shown efficacy with increased production more than 300 fold over the baseline in mice. Even though the concentration of hBChE showed protection up to 5 LD₅₀ of VX in mice, it was not long lasting (~10 days) due to adenovirus immunogenicity (69–71). An alternative strategy arose with adeno-associated virus vectors (AAV) to produce hBChE after it was regarded as safe (72). The AAV vector used in bicistronic form to co-express hBChE and poly-

prolin peptide facilitate the formation of fully functional tetramers. The administration of AAV-hBChE vectors with intra muscular injection provided expression of hBChE within 3–4 weeks in mice similar as in studies with intravenous injection for delivery of AVV vectors to the liver. From the liver, expressed enzyme levels were sustained for 8–16 months, but from muscles after single injection expression lasted without silencing up to 140 days (73–75). Levels of expression achieved through 2–3 weeks were sufficient to neutralize 2 LD₅₀ VX. Taking into consideration the correlation of inter muscular dose of 10¹² genome copies per mouse and 0.5 mg/ml of active hBChE in serum, prophylactic efficiency can be adjusted by vector dose (75). The results obtained so far are encouraging for further studies on non-human primates to ascertain the AVV-mediated delivery efficiency and impact on the immunosystem with higher doses.

Recently Gao *et al.* (76) showed that the AAV gene transfer of BChE could impact BChE expression across the entire brain or in selective regions of the central nervous system. Their approach was focused on the BChE gene transfer as a means to explore the emerging issue of the enzyme's physiologic role as a key regulator of ghrelin in general and, more particularly, in specific brain centers involved in emotional states that are strongly influenced by this peptide hormone (76).

CONCLUSION

Butyrylcholinesterase, even though without any essential function in the organism, has shown great importance as a bioscavenger for cholinergic systems in cases of OP exposure. One drawback to bioscavenger-based therapy is the need for sustained delivery of large enzyme quantities - an expensive process. To alleviate this problem, recombinant technology, expression system and more recently gene therapy made a major breakthrough in pursuance of economic goals, requirements for post-modification and also to gain long-term systematic expression of fully functional hBChE in efficacious levels. In regard with expression system, gene therapy could also show potential in unresolved issues of BChE's physiological roles or its role in the onset and progression of Alzheimer's disease.

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