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Amino acid residues at the N- and C-termini are essential for the folding of active human butyrylcholinesterase polypeptide

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ABSTRACT

Human serum butyrylcholinesterase (HuBChE) is currently the most suitable bioscavenger for the prophylaxis of highly toxic organophosphate (OP) nerve agents. A dose of 200 mg of HuBChE is envisioned as a prophylactic treatment that can protect humans from an exposure of up to 2 × LD_{50} of soman. The limited availability and administration of multiple doses of this stoichiometric bioscavenger make this pretreatment difficult. Thus, the goal of this study was to produce a smaller enzymatically active HuBChE polypeptide (HBP) that could bind to nerve agents with high affinity thereby reducing the dose of enzyme. Studies have indicated that the three-dimensional structure and the domains of HuBChE (acyl pocket, lip of the active center gorge, and the anionic substrate-binding domain) that are critical for the binding of substrate are also essential for the selectivity and binding of inhibitors including OPs. Therefore, we designed three HBPs by deleting some N- and C-terminal residues of HuBChE by maintaining the folds of the active site core that includes the three active site residues (S198, E325, and H438). HBP-4 that lacks 45 residues from C-terminus but known to have BChE activity was used as a control. The cDNAs for the HBPs containing signal sequences were synthesized, cloned into different mammalian expression vectors, and recombinant polypeptides were transiently expressed in different cell lines. No BChE activity was detected in the culture media of cells transfected with any of the newly designed HBPs, and the inactive polypeptides remained inside the cells. Only enzymatically active HBP-4 was secreted into the culture medium. These results suggest that residues at the N- and C-termini are required for the folding and/or maintenance of HBP into an active stable, conformation.

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1. Introduction

Plasma-derived cholinesterases (ChEs) are effective bioscavengers of highly toxic organophosphorus compounds (OP) including pesticides and nerve agents [1]. Exogenously administered plasma-derived human butyrylcholinesterase (HuBChE) protects animals from multiple LD_{50}s of OP nerve agents without any toxic effects or performance decrements [2–4]. Thus, HuBChE is currently in advanced development as a prophylactic treatment for nerve agent toxicity. A dose of 200 mg of HuBChE is estimated to protect humans from an exposure of up to 2 × LD_{50} of soman [5]. However, the production of native HuBChE in such quantities is expensive and requires large amounts of plasma. This limited availability and administration of multiple doses of large quantities of this stoichiometric bioscavenger make this pretreatment difficult.

One approach for reducing the dose of this enzyme is to design a small molecule polypeptide bioscavenger that can be produced in large quantities using molecular biology techniques. HuBChE is a globular protein made up of four identical polypeptides of 574 amino acids each. Previous studies have shown that deletion of 45 C-terminal amino acids resulted in secretion of active HuBChE monomers [6]. Therefore, in this study, the production of smaller HuBChE polypeptides (HBPs) was attempted by deleting more amino acid residues from both N- and C-termini. The active site S198 in HuBChE is also the site for the binding of OPs. This was clearly demonstrated by isolating a HuBChE nonapeptide (FGESAGAAS)

Abbreviations: AChE, acetylcholinesterase; BHK, baby hamster kidney cells; BTC, butyrylthiocholine iodide; ChE, cholinesterase; CHO, Chinese hamster ovary cells; DTNB, 5,5′-dithiobis 2-nitrobenzoic acid; HBP, HuBChE polypeptide; HEK, human embryonic kidney cells; HuBChE, human butyrylcholinesterase; OP, organophosphorus compounds.

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that includes the active site Ser modified by various OP and non-OP inhibitors [7]. Results of site-directed mutagenesis, molecular modeling, and X-ray crystallography studies suggest that the architecture of the active-site gorge is important for the binding of OPs to ChEs. The structural features of HuBChE that maintain the folds of the active site are contained in the polypeptide region between residues 61 and 478 of HuBChE [8–12]. Accordingly, three HBPs that lack amino acid residues from either N- or C-terminus (HBP-1$^{(1-478)}$, HBP-2$^{(61-529)}$, HBP-3$^{(156-529)}$) were designed. HBP-4$^{(1-529)}$ that lacks 45 residues from C-terminus but known to have BChE activity was used as a control. The cDNAs for the HBPs containing signal sequences were synthesized and cloned into different mammalian expression vectors, which were used for transfecting different cell lines. BChE activity secreted into the culture medium and in cell lysates was tested.

2. Materials and methods

2.1. Reagents and cells

Gene juice, nano juice and pTriEx-4Neo plasmid were purchased from EMD Millipore Chemicals (San Diego, CA). pcDNA™3.1/V5-His TOPO TA expression kit, Lipofectamine 2000, Lipofectamine Plus, Lipofectamine LTX Plus and 293fectin transfection reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). Plasmid containing HuBChE (pGS-HuBChE) gene was generous gift from Dr. Oksana Lockridge (University of Nebraska, Omaha, NE). Rabbit polyclonal antibodies to purified plasma-derived HuBChE were produced by Spring Valley Laboratories, Inc., Woodline, MD.

2.2. Designing of HBPs

Molecular dynamics simulations and X-ray crystal structure analysis of HuBChE show that the structural features of HuBChE that are required for effective binding of OPs are largely contained in the 61–478 polypeptide region. The essential amino acid residues involved in OP-binding are contained in five peptide fragments (61–88, 108–148, 187–236, 285–336, and 384–478) [11–12]. These peptides were identified by deleting residues surrounding the active site while still maintaining its architecture. The resulting molecule contained essential binding subsites: (1) three-pronged oxyanion hole, (2) pi-cation site, (3) acyl-binding pocket, (4) gorge rim residues, (5) E197 that makes hydrogen bonds directly or through water molecules with residues at the catalytic site, and (6) D70, that plays an important role in promoting substrate binding and in controlling the dynamics and structural organization of water molecules. Based on this information, (1) HBP-1 was designed by deleting 96 amino acid residues from C-terminus (479–574), (2) HBP-2 was designed by deleting 60 amino acid residues from N-terminus (1–60) and 45 amino acid residues from C-terminus (530–574), (3) HBP-3 was designed by deleting 155 residues from N-terminus (1–155) and 45 amino acid residues from C-terminus (530–574) of the HuBChE protein (Figs. 1 and 2). HBP-4 that lacks 45 residues from C-terminus (530–574) was used as a control.

2.3. Cloning and expression of recombinant HBPs

Genes encoding HBPs containing the signal peptide and Kozak sequence at their N-terminus were synthesized (Retrogen, Inc., San Diego, CA). A signal peptide that drives the secretion of

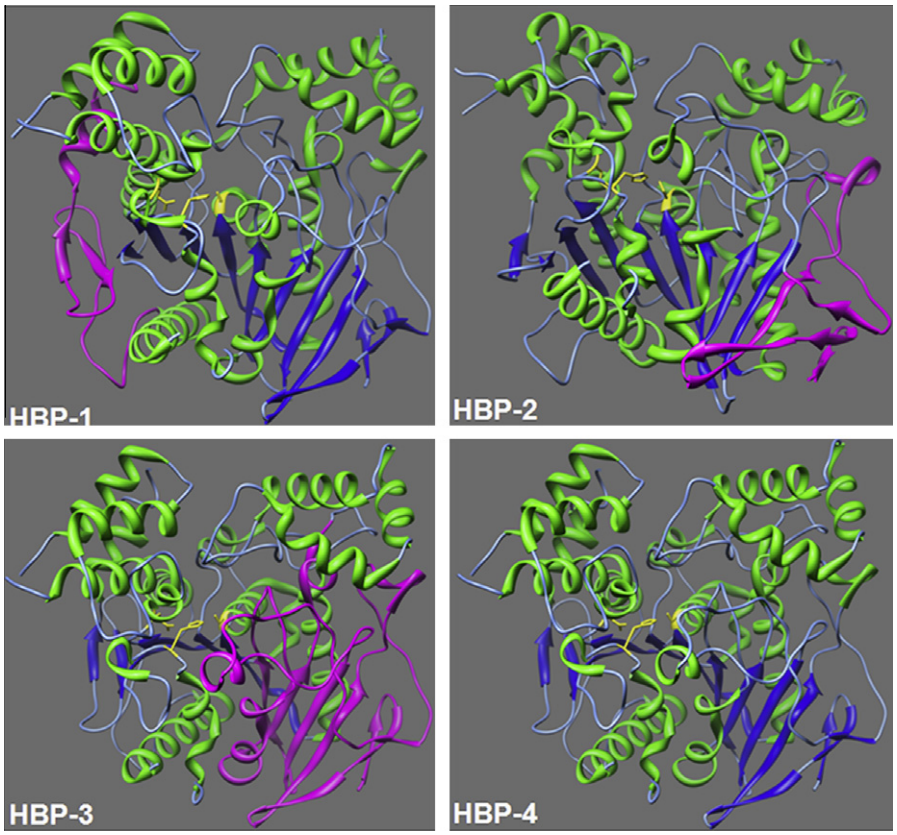

Fig. 1. Ribbon diagrams or predicted structures of HBPs. Sequences were selected based on the X-ray crystal structure of HuBChE (PDB ID: 1P0I), and N- and C-terminus regions forming small self-folded domains and predicted to be not required for HuBChE activity were deleted (shown in purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

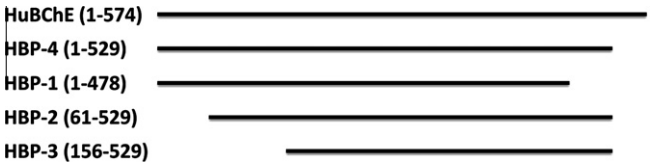

Fig. 2. Relative sizes of designed HBPs after deleting N- and C-terminal sequences of HuBChE.

full-length HuBChE into the culture medium [13] was used in this study. Suitable restriction enzyme sites were placed at the 5′- and 3′-ends of these genes so that they could be cloned into any mammalian expression vector. To generate pTriEx-4Neo- and pcDNA3.1-HBP expression vectors, HBP inserts were created by digestion of pCR-Blunt-HBP plasmids with BamHI and NotI, and the gel-purified inserts were ligated with BamHI and NotI-digested vectors (Fig. 3). To generate pGS-HBP expression vectors, the HBP genes prepared by gel-extraction following digestion of pCR-Blunt-HBP plasmids with HindIII and ApaI, were subcloned into HindIII and ApaI-digested pGS vector. The sequences of HBPs in all the expression vectors were verified by DNA sequencing.

The expression of HBPs by the constructs in different vectors was investigated in various cell lines using transient transfection. Plasmid DNAs were introduced into HEK-293A, BHK or CHO cells using different transfection reagents according to the manufacturer's protocols, and the culture medium was assayed for BChE activity at 24, 48 and 72 h. Cell lysates were prepared by removing culture medium from cells at 72 h after transfection and adding 0.2 ml of Cytobuster protein extraction reagent (Novagen, Madison, WI) to the wells. Following incubation at 22 °C for 5 min, lysates were centrifuged at 16,000*g* for 15 min, and clear supernatants were assayed for BChE activity.

2.4. Determination of enzyme activity of HBPs

Enzyme activity of HBPs was measured by the Ellman assay [14] in 50 mM sodium phosphate buffer, pH 8.0, at 22 °C, in the presence of 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), using 1 mM butyrylthiocholine iodide (BTC) as the substrate.

2.5. Western blot analysis of HBPs

Proteins in culture media or cell lysate separated on a 12% polyacrylamide gel under denaturing and reducing conditions [15] were transferred to a PVDF membrane [16]. After incubation with 4% nonfat dry milk powder in 50 mM Tris buffer, pH 7.6, 150 mM NaCl, 0.05% Tween 20, membranes were incubated with polyclonal anti-HuBChE antibody. Proteins were detected by incubation with a 1:100,000-diluted horseradish peroxidase-conjugated secondary anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) and using an ECL Plus chemiluminescence detection kit (GE Healthcare Life Sciences, Piscataway, NJ).

3. Results and discussion

HuBChE is a stoichiometric bioscavenger that can bind to and neutralize all OP nerve agents. However, a large dose of HuBChE is needed to counteract the toxic effects of OPs. Therefore, in order to reduce the dose, smaller OP-binding polypeptides were designed by deleting N- and C-terminus regions of HuBChE (Figs. 1 and 2). These deleted sequences form small self-folded flexible domains and were predicted to be not required for HuBChE activity. HBPs of different lengths and sequences were synthesized and cloned into various mammalian expression vectors (pcDNA3.1, pTriEx-4Neo, and pGS) in order to select the best vector for the expression of HBPs. HBP inserts in pcDNA3.1 plasmid following digestion with BamHI and NotI are shown in Fig. 3. All the HBP inserts were of correct size and in right orientation. Similarly, the sizes, orientation and sequences of HBP inserts in other expression vectors were verified (data not shown). Preliminary studies showed that pcDNA3.1 and pGS plasmids, compared to pTriEx-4Neo, were better expression vectors for expressing HBP-4 or HuBChE (data not shown).

The next step was to determine the best transfection reagent, optimum ratio of DNA to transfection reagent and the best cell line for the expression of HBPs. This was achieved by using varying amounts of transfection reagents for transfecting different cell lines with 2 μg of pcDNA3.1-HBP-4 and assaying supernatants for BChE activity. As shown in Fig. 4, levels of BChE activity expressed by CHO-K1 cells (ATCC# CCL-61), was in the following order: Lipofectamine LTX Plus > Lipofectamine 2000 > 293fectin > Gene juice > Lipofectamine Plus > Nano juice. Results of similar evaluations conducted in other cell lines are summarized as follows. For CHO-K1 cells (ATCC# CRL-9618): Lipofectamine LTX Plus > Lipofectamine 2000 > Gene juice > 293fectin > Lipofectamine Plus > Nano juice. For BHK50 cells (ATCC# CRL-10314): Lipofectamine 2000 > 293fectin > Lipofectamine Plus = Lipofectamine LTX Plus > Gene juice > Nano juice. For BHK-21 cells (ATCC# CCL-10): Lipofectamine 2000 > 293fectin > Lipofectamine Plus = Lipofectamine LTX Plus > Gene juice > Nano juice. For HEK-293A cells: Lipofectamine 2000 > 293fectin > Lipofectamine LTX Plus > Lipofectamine Plus > Gene juice > Nano juice. Based on these results, Lipofectamine 2000 with a ratio of 1:2 (DNA:Lipofectamine

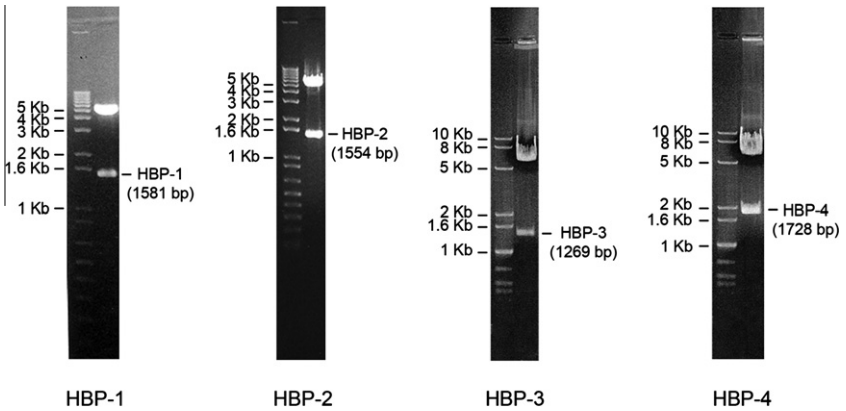

Fig. 3. HBPs in pcDNA3.1 plasmid. pcDNA3.1-HBP plasmids were treated with BamHI and NotI, electrophoresed on 1% agarose gel and stained with ethidium bromide.

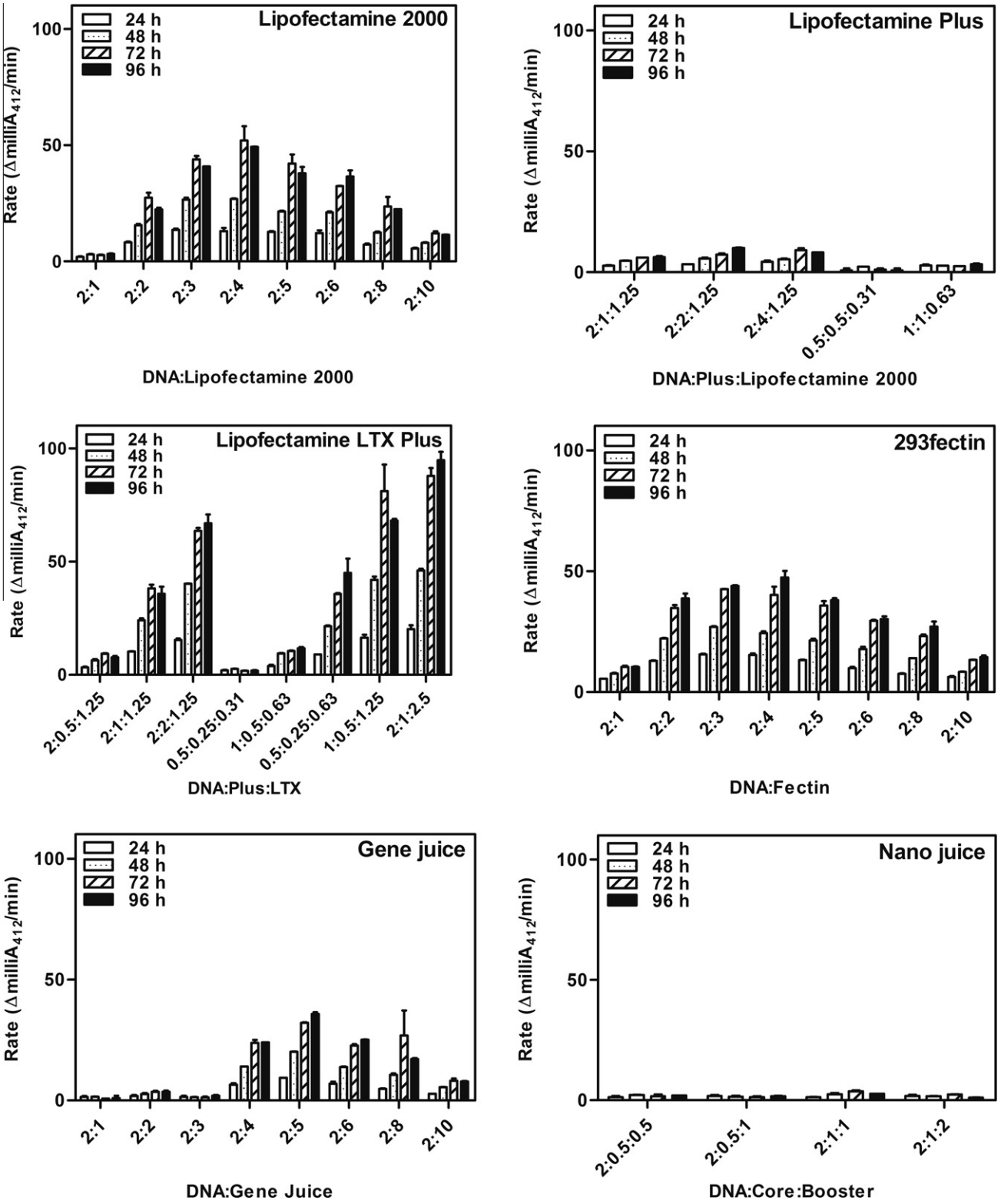

Fig. 4. Expression of HBP-4 in CHO-K1 cells. CHO-K1 cells (ATCC# CCL-61), grown overnight in 12-well plates were transfected with 2 μg of pcDNA3.1-HBP-4 plasmid DNA using various transfection reagents, according to the manufacturer's protocols. BChE activity in the culture medium was measured by the Ellman assay [14], using 1 mM BTC as substrate. Numbers on *X*-axis indicate the ratios of DNA (μg) to transfection reagent components (μl).

2000) and either HEK-293A or CHO-K1 (ATCC# CCL-61) cells were selected for the expression of HBPs.

HEK-293A cells transfected with pcDNA3.1 vectors containing HBPs 1, 2, 3 did not secrete any BChE activity into the culture medium (Fig. 5). Similarly, HEK-293A cells transfected with pTriEx-4Neo-HBPs and CHO-K1 cells transfected with pcDNA3.1- and pGS-HBPs did not secrete any BChE activity into the culture medium (data not shown). On the other hand, BChE activity was secreted by all cell lines transfected with expression vectors containing HBP-4 (Figs. 4 and 5). These results indicate that the

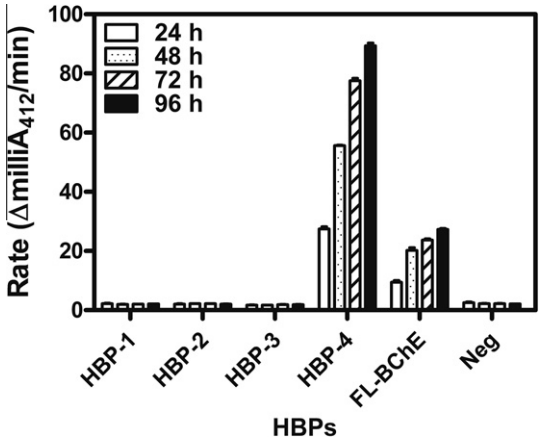

Fig. 5. Expression of HBPs in HEK-293A cells. Cells grown overnight in 12-well plates were transfected with 2 μg of pcDNA3.1-HBP plasmid DNAs and 4 μl of Lipofectamine 2000. BChE activity in the culture medium was measured by the Ellman assay [14]. FL-BChE represents full length HuBChE positive control and Neg, Lipofectamine 2000 negative control.

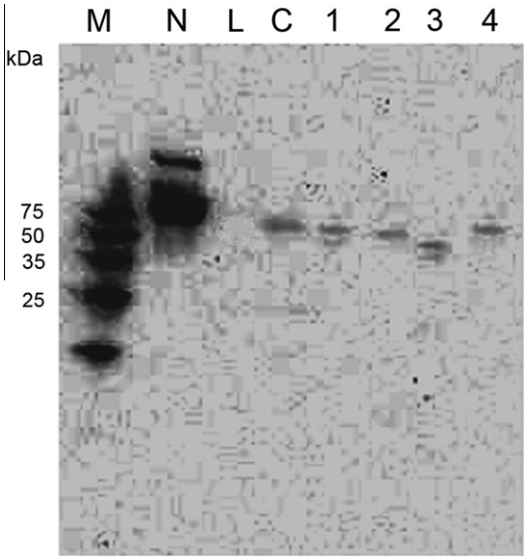

Fig. 6. Western blot analysis of HBPs in cell lysates. HEK-293A cells were transfected with pcDNA3.1-HBP plasmid DNAs. After 72 h of transfection, culture medium was removed and cells were lysed with SDS PAGE reducing sample buffer. Following SDS PAGE on a 12% gel, proteins were transferred to a PVDF membrane, and HBPs were detected using polyclonal rabbit anti-HuBChE antibodies. M, Millipore perfect protein Western markers, N, native plasma-derived HuBChE; L, lipofectamine negative control, C, full-length HuBChE positive control; 1–4 represent HBPs 1–4.

deletion of 45 amino acid residues from HuBChE C-terminus that form the tetramerization domain does not adversely affect the expression, secretion and enzymatic activity of HuBChE, which is consistent with previous observations [6]. On the contrary, as previously noted [6], this deletion resulted in increased expression and secretion of monomers into the culture medium (HBP-4, Fig. 5). However, the deletion of additional 51 amino acids from HBP-4 or 96 amino acids from the HuBChE C-terminus (HBP-1) completely abolished the secretion of active HuBChE into the culture medium. Similarly, the deletion of even 60 amino acid residues from the N-terminus (HBP-2) had a severe effect on the secretion of active HuBChE into the culture medium.

To determine if deletions of amino acid residues at the N- and C-termini affected activity of expressed polypeptides or their secretion into culture media, cell lysates of HEK-293A cells transfected with pcDNA3.1-HBPs-1, 2, 3 expression vectors were also assayed for BChE activity. No BChE activity was detected in cell lysates. Furthermore, both culture media and cell lysates of HEK-293A cells transfected with pcDNA3.1-HBPs-1, 2, 3 expression vectors were subjected to Western blotting. As shown in Fig. 6, BChE polypeptides were detected in cell lysates, suggesting that inactive polypeptides were retained in cells. No HBPs were detected in the culture media (data not shown). Thus, truncation of residues at N- and C-termini affect the activity as well as secretion of expressed HBPs.

Our observations corroborate the findings of previous studies, which attempted to produce truncated HuBChEs and acetylcholinesterases (AChEs). Blong et al. [6] showed that deletion of 51 or more amino acids from the C-terminus of HuBChE destroyed BChE activity and caused the inactive protein to remain in the cell. Deletion of 8 or more amino acids from the N-terminus also resulted in inactive protein that remained inside the cell [6]. Similarly, a truncated AChE that lacks the C-terminal 40 amino acids was secreted into the medium, whereas the mutant AChE that lacks 65 amino acids from C-terminus was inactive, suggesting that these domains contribute to proper folding and catalytic activity [17]. By measuring both catalytic activity and isotopic labeling, pulse-chase studies showed that only the catalytically active molecules are further processed by the cell, whereas the inactive molecules are rapidly degraded intracellularly [18]. In conclusion, studies conducted with HuBChE suggest that the inactivity of the designed HBPs is likely due to incorrect folding, and the amino acid residues at both the N- and C-termini are required for the folding and/or maintenance of HBP into an active, stable conformation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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