Biochemical Pharmacology, Vol. 60, pp. 121–126, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00238-0

High-Level Expression of Human Butyrylcholinesterase Gene in *Bombyx mori* and Biochemical-Pharmacological Characteristic Study of Its Product

Wan-Li Wei, Jun-Chuan Qin† and Man-Ji Sun*‡*

*Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing 100850; and †National Key Laboratory of Pharmaceutical Biotechnology, Nanjing 210093, P. R. China

ABSTRACT. The human butyrylcholinesterase (BChE, EC 3.1.1.8) gene was highly expressed in *Bombyx mori* using baculovirus vector, and the biochemical-pharmacological properties of its product were studied. BChE cDNA was cloned into transfer vector pBn96 and co-transfected with wild-type *Bombyx mori* nucleopolyhedrovirus (BmNPV) DNA into BmN cells. The recombinant virus with the highest enzyme activity was sorted out and purified. Once the BmN cells or silkworm larvae had been infected with the recombinant virus, recombinant human BChE (rhBChE) could be secreted into the culture medium or the hemolymph of the larvae at levels of 1.5 mg $\cdot L^{-1}$ and 35 mg $\cdot L^{-1}$, respectively. Western blot and enzymatic staining of the electrophoresis gel of non-denatured protein showed that rhBChE manifested similar antigenicity and enzyme activity to native human BChE (nhBChE). The production of rhBChE in the hemolymph was 23-fold higher than that in BmN cells and about 280-fold that in Chinese hamster overy cells (125 $\mu g \cdot L^{-1}$). This is the first report of human BChE expression in silkworm with the highest level of yield so far. rhBChE was highly similar to nhBChE in respect to substrate affinity, inhibitor sensitivity, and reactivity of the inhibited enzyme. It is suggested that rhBChE functions as well as nhBChE and has potential practical value. BIOCHEM PHARMACOL **60**;1:121–126, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. butyrylcholinesterase; gene expression; baculovirus; *Bombyx mori*; sarin

Butyrylcholinesterase is distinguished from acetylcholinesterase by substrate specificity and inhibitor sensitivity. BChE§ is able to combine with organophosphorous nerve agents and insecticides and to hydrolyze many ester-containing drugs such as cocaine and succinylcholine [1, 2]. Therefore, BChE may serve as a natural scavenger for a variety of drugs, poisons, and organophosphorous compounds. It has been demonstrated that administration of exogenous BChE can prevent mouse, rat, and monkey from multiple lethal-dose organophosphate intoxication [3, 4]. A sufficient amount of exogenous BChE, which maintains a high level of BChE in the circulating blood, is also efficient in therapy of dyspnea or apnea caused by succinylcholine, a muscle relaxant in surgery. Considering the potential pharmaceutical importance of BChE, attempts have been made to produce BChE in many expression systems such as *Escherichia coli*, *Xenopus laevis* oocytes, and CHO mammalian cells. However, rhBChE exhibited little or low enzyme activity and production [5–7]. It has been documented that the newly established expression system in *Bombyx mori* utilizing the strong promoter of the gene encoding the polyhedron protein in the BmNPV could express high-yield foreign proteins with biological activities comparable to those of their native counterparts. In this study, nhBChE cDNA was expressed in cultured cells of *B. mori* and silkworm larvae using the baculovirus expression system. The biochemical-pharmacological properties of the rhBChE were also studied.

MATERIALS AND METHODS

Materials

Restriction endonucleases, Klenow fragment of DNA polymerase, and T_4 DNA ligase were obtained from New England Biolabs. The Digoxigenin DNA labelling and detection kit was purchased from Boehringer Mannheim. Insect cell culture medium TC-100 was from GIBCO BRL. Rabbit antihuman serum BChE polyclonal antibodies were from DAKO Labs. Horseradish peroxidase-conjugated sheep antirabbit IgG was from Sino-American Biotechnol-

‡ Corresponding author: Dr. Man-Ji Sun, Institute of Pharmacology and Toxicology, Tai-Ping Road 27, Beijing 100850, China. FAX (6810) 68211656; E-mail: Sunmj@nic.bmi.ac.cn

§ *Abbreviations:* BChE, butyrylcholinesterase; hBChE, human butyrylcholinesterase; rhBChE, recombinant human butyrylcholinesterase; nhBChE, native human butyrylcholinesterase; BmNPV, *Bombyx mori* nucleopolyhedrovirus; CHO, Chinese hamster ovary; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; HI-6, 1-(2-hydroxyimino methyl-1-pyridinio)-3-(4-carbamoyl-1-pyridinio)-2-oxapropane dichloride; Ph, polyhedrin promoter; P_{CMV}, cytomegalovirus promoter; and IgG, immunoglobulin G.

Received 19 May 1999; accepted 14 October 1999.

ogy Company. DTNB and reactivator HI-6 were synthesized by the Department of Chemical Synthesis in our Institute. Silkworm larvea, Su5× Su6 species, were obtained from the Silkworm Institute, Chinese Academy of Sciences. Plasmid pRc CMV–BChE was a gift from Dr. O. Lockridge. The high efficiency transfer vector pBn96 was constructed in our laboratory.

Cells and Viruses

Silkworm *B. mori* (BmN) cells provided by the National Key Laboratory of Pharmaceutical Biotechnology were cultured in TC-100 with 10% bovine serum. Wild-type BmNPV DNA was isolated as previously reported from the hemolymph of silkworm larvae that had been inoculated with wild-type BmNPV [8].

Construction of Recombinant Baculovirus

The entire nhBChE cDNA signal and coding sequence (1.8 kb) was excised from plasmid pRc CMV–BChE by Bgl II digestion, filled in with Klenow fragment, and ligated at the Sma I site of the transfer vector pBn96. In the resulting pBn96–BChE vector, nhBChE cDNA inserted in the same direction of the Ph was identified by *Eco*RI + *Bam*HI digestion with 8.1-, 1.7-, and 1.3-kb fragments (Fig. 2, lane 2), whereas nhBChE cDNA inserted in the reverse direction exhibited 8.1-, 1.3-, and 0.1-kb fragments. The pBn96 vector which failed in insertion gave two fragment bands, 8.1 and 1.3 kb. We found only the clone of *cis*-inserted hBChE cDNA. The resulting pBn96–BChE plasmid was co-transfected (mediated by lipofectin) with wild-type BmNPV DNA into BmN cells. The viral progeny were screened for the presence of recombinant virus in which the polyhedrin gene was inactivated by the insertion of nhBChE cDNA. The recombinant virus was identified by dot-blot hybridization using a digoxigenin-labeled fragment of BChE cDNA as probe and checked for BChE activity.

BChE Assay

The enzyme in 0.1 mol · L^{-1} sodium phosphate buffer (PBS, pH 7.4) reacted with 1.5 mmol · L^{-1} butyrylthiocholine in a total volume of 200 μL at 37° for 30 min, then 50 μL of 0.08% (w/v) DTNB was added and the absorbance at 414 nm was monitored. BChE activity and residual activity after inhibition and reactivation were calculated according to the calibration curve of cysteine reacting with DTNB.

Time–Course of BChE Expression

The BmN cells were seeded into flasks and the recombinant virus inoculum was added. The silkworm larvae were infected with the purified recombinant virus by subcutaneous injection. The supernatant of the medium and the hemolymph of the larvae containing the secreted hBChE were collected at various intervals and investigated for enzyme activity.

SDS–PAGE and Western Blot

SDS–PAGE was performed under reducing and non-reducing conditions at 20 mA for 4 hr. The 1.5-mm-thick gel was made up of 8% (w/v) separating gel and 5% (w/v) stacking gel. Then, the gel was electrophoretically transferred onto nitrocellulose membrane [8]. Immunoblot analysis was carried out according to the protocol (Bio-Rad, Bulletin 1721, Catalog No. 170-3980) using fat-free milk for blocking and rabbit antihuman serum BChE polycolonal antibodies and horseradish peroxidase-conjugated sheep antirabbit IgG antibody as the detecting reagents.

Non-Denatured Polyacrylamide Electrophoresis

Non-denaturing gels (1.5-mm thick) were made up of 6% (w/v) separating gel and 5% (w/v) stacking gel layers. Electrophoresis was carried out in 25 mmol · L^{-1} Tris–250 mmol · L^{-1} glycine buffer at 100 V for 6 hr. The gel was developed in BChE activity-staining buffer as described [9].

Inhibition of hBChE Activity by Sarin

Aliquots of 20 μL of 1:1000 diluted nhBChE or 50 μL of 1:400 diluted hemolymph containing rhBChE were reacted with various concentrations of sarin diluted with 0.1 mol · L^{-1} PBS (pH 7.4) in a total of 150 μL, incubated at 37° for 10 min, and then the residual BChE activity was determined. Inhibition rates were expressed as percentages of activity added.

Reactivation of Sarin-Phosphonylated hBChE

Sarin-inhibited nhBChE or rhBChE was reactivated by 0.1 mmol · L^{-1} HI-6 in 0.1 mol · L^{-1} PBS (pH 7.4) at 37° for various lengths of time, then BChE activity was determined. Controls without reactivator and/or enzyme were set synchronously. Reactivation rates were calculated according to Child's formula: [(EIR-EI)/(E-EI × 100%].

Inhibition of BChE by Rabbit Antihuman Serum BChE Polyclonal Antibody

rhBChE 1:400 and nhBChE 1:1000 were mixed with various concentrations of diluted rabbit antihuman serum BChE polyclonal antibody against human serum BChE at 4° for 4 hr. Then, the residual BChE activity was assayed.

RESULTS

Construction of the Recombinant Baculovirus BmNPV–BChE

The protocol for the construction of the pBn96 transfer vector carrying hBChE cDNA is shown in Fig. 1. hBChE

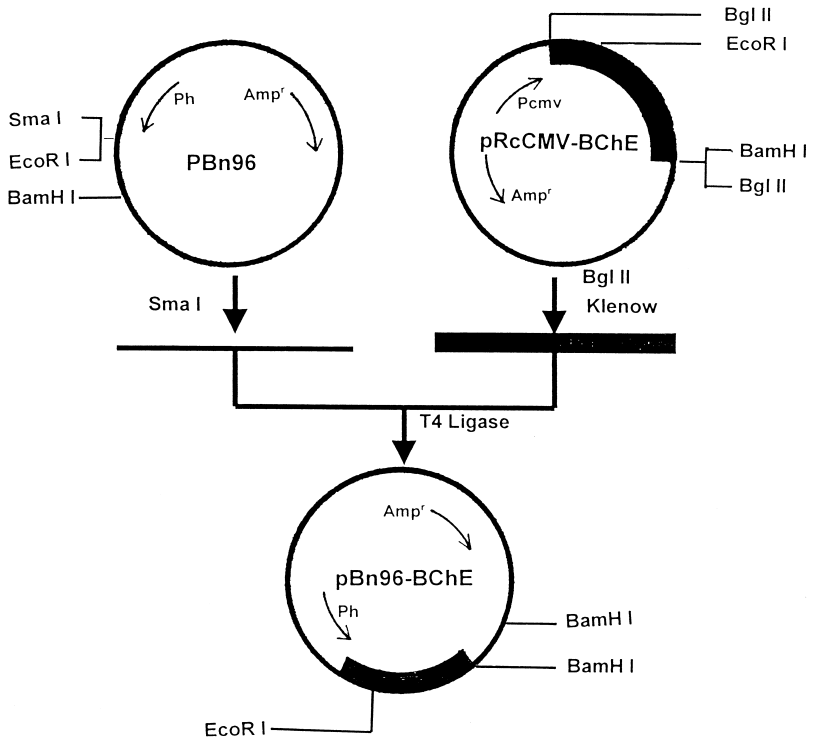

FIG. 1. Construction of recombinant transfer vector pBn96–BChE. Details are given in Methods and Results. Amp^r, ampicilin resistance.

cDNA (1722 bp) bearing an 840-bp signal peptide sequence was excised from the plasmid pRc CMV–hBChE by Bgl II digestion, blunted, and cloned into the Sma I site of the pBn96 transfer vector. The recombinant plasmid pBn96–hBChE in which the hBChE gene was oriented from the 5′ to the 3′ terminus, i.e. the same direction as the promoter of the polyhedrin gene, was sorted out by checking the 8.1-, 1.7-, and 1.3-kb segments of *Eco*RI + *Bam*H I cutting on the restriction map (Fig. 2, lane 2). The recombinant plasmid was co-transfected with wild-type BmNPV DNA into BmN cells. The recombinant virus was identified and purified. The viral progeny were screened for the presence of recombinant virus in which the polyhedrin gene was inactivated by the insertion of hBChE cDNA. Eight positive recombinant viruses were screened. One of the viruses with the highest biological activity (BmNPV–BChE) was chosen for further study.

Expression of rhBChE in BmN Cells and Silkworm Larvae

The BmN cells and silkworm larvae were infected with the purified recombinant virus for expression of hBChE. rhBChE activity in the culture supernatant and hemolymph was detected separately 24 hr after infection. A time–course study showed that the activity of BChE reached its maximum level at the fourth day postinfection (Fig. 3). The highest activity of rhBChE in the hemolymph was 24.7 units/mL, which is equivalent to 35 μg/mL, and significantly higher than that secreted into the BmN cell culture medium (1.1 units/mL). Generally, 0.7 mL of hemolymph can be squeezed out from one silkworm larva. Thus, 25 mg of rhBChE could be harvested from 1000 larvae. The yield of enzyme *in vivo* was 23-fold higher than that in BmN cells *ex vivo*.

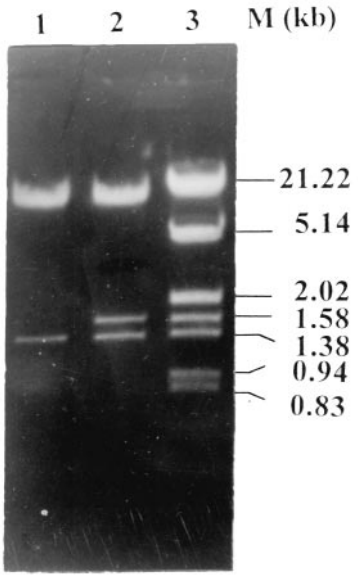

FIG. 2. Identification of recombinant plasmid pBn96–BChE. Lane 1: plasmid pBn96 digested with *Eco*R I + *Bam*H I (8.1 and 1.3 kb). Lane 2: plasmid pBn96–BChE digested with *Eco*R I + *Bam*H I (8.1, 1.7, and 1.3 kb). Lane 3: DNA markers (λDNA + *Eco*R I + *Hind* III).

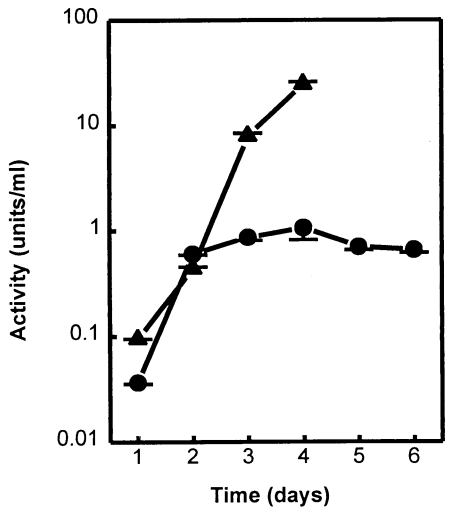

FIG. 3. Time–course of rhBChE expression in BmN cells and silkworm larvae. After BmNPV–BChE recombinant virus infection, the supernatant of cell culture media and the hemolymph of the silkworm larvae were collected separately for enzyme activity assay at 24-hr intervals. BmN cells: 10^6 cells/mL, mean values (●) ± SD, N = 3 independent experiments in duplicate; silkworm larvae: mean values (▲) ± SD, N = pooled hemolymph from five larvae in a typical experiment.

The protein bands were combined well with the anti-BChE polyclonal antibody for immunoblot analysis (Fig. 4). rhBChE showed two monomer bands with molecular weights of 65 and 71 kDa under reducing conditions according to the molecular weight migration map (Fig. 4, lane 2), much smaller than that of the nhBChE monomer (85 kDa). Glycoside cutting showed that the different molecular weights were due to the difference in glycosylation between silkworm and human (Fig. 5). Glycosylation

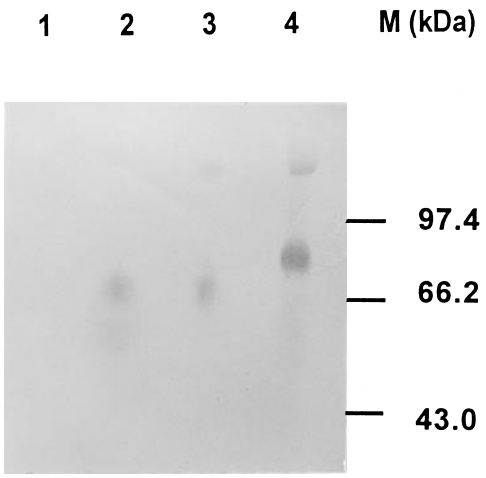

FIG. 4. Western blot analysis of rhBChE produced by BmN cells. Lane 1: control, the medium of BmN cells infected with wild-type BmNPV. Lane 2: rhBChE in the medium of cells infected with BmNPV–BChE under reducing conditions. Lane 3: rhBChE in the medium of cells infected with BmNPV–BChE under non-reducing conditions. Lane 4: human serum cholinesterase under non-reducing conditions. Lane 5: protein molecular weight markers.

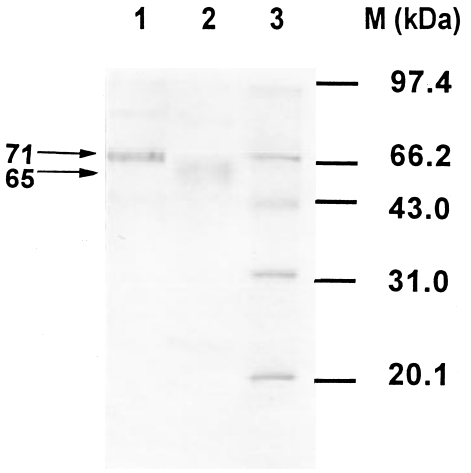

FIG. 5. SDS–PAGE of rhBChE deglycosylated by N-glycosidases F. SDS–PAGE (8% separating gel and 5% stacking gel) was performed under reducing conditions. Lane 1: partially purified rhBChE without deglycosylation (71-kDa monomer). Lane 2: partially purified rhBChE with N-glycosidases F (65-kDa monomer). Lane 3: protein molecular weight markers.

does not appear to be required for BChE activity [5]. The electrophoresis gel running under non-denatured conditions was obviously stained for the BChE activity. All the above-mentioned results imply that rhBChE is efficiently secreted into the hemolymph of silkworm larvae as well as into the culture medium of BmN cells (Fig. 6). The Lineweaver–Burk reciprocal plot showed that the K_m and V_{max} values of rhBChE were 0.185 mmol · L^{-1} and 1.81 nmol · L^{-1} · min when butyrylthiocholine was used as substrate, similar to those of nhBChE (0.181 mmol · L^{-1} and 1.86 nmol · L^{-1} · min).

Inhibitor Sensitivity of hBChE

The inhibitor sensitivities of nhBChE and rhBChE to sarin were quite similar (Fig. 7). About 90% of the activities of

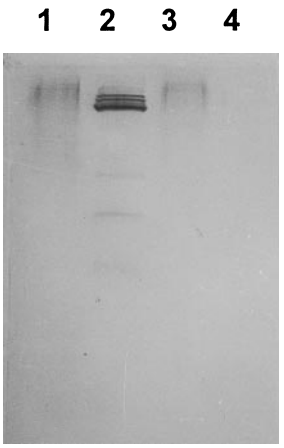

FIG. 6. Non-denaturing gel stained for BChE activity. Lane 1: 20 μL of 1:20 diluted larvae hemolymph infected with BmNPV–BChE. Lane 2: 20 μL of human serum cholinesterase. Lane 3: 20 μL of BmN cell supernatant infected with BmNPV–BChE. Lane 4: 20 μL of 1:20 diluted larvae hemolymph infected with wild-type BmNPV.

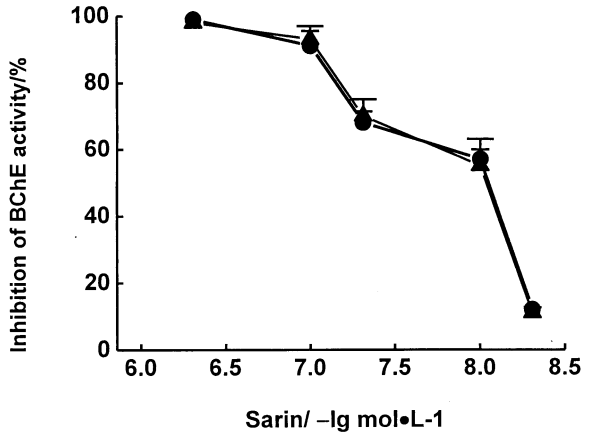

FIG. 7. Sarin inhibition curve of BChE. rhBChE (●) and nhBChE (▲). N = 3 independent experiments in duplicate, mean values ± SD; −log denotes the negative logarithm of molarity of sarin.

rhBChE and nhBChE were inhibited by 100 nmol · L^{-1} of sarin with a pI_{50} value of 8.2 and pI_{90} value of 7.0.

Reactivation of Inhibited BChE by HI-6

Sarin-phosphonylated BChE was reactivated spontaneously or by incubation with HI-6 in a time-dependent manner. The reactivation rates of inhibited rhBChE and nhBChE in spontaneous and HI-6 reactivation were comparable (Table 1).

Inhibition of BChE by Rabbit Antihuman Serum BChE Polyclonal Antibody

rhBChE and nhBChE were immunoreacted with the polyclonal antibody separately for 4 hr at 4°. It could be observed that the enzyme activities decreased with the increase in rabbit antihuman BChE IgG (Table 2). The inhibition rates of rhBChE fell *pari psasu* that of nhBChE.

DISCUSSION

hBChE cDNA encodes an enzyme polypeptide of 574 amino acids and a signal peptide of 28 amino acids. There are nine *N*-glycosylation sites and eight cysteine residues altogether. Six cysteine residues form three intrachain disulfide loops (Cys 65–92, 252–263, and 400–519). Cys 571 forms an interchain disulfide bond with Cys 571 of an identical subunit. The 574 amino acids in the subunit of hBChE have a calculated molecular weight of 65,092. Fully glycosylated hBChE gives a total weight of 85,534. The tetramer is composed of four identical subunits assembling as a dimer of dimers with a molecular weight of 342,136 [2]. The complexity of the hBChE structure made difficult the high-level expression in *Escherichia coli*, oocytes of *Xenopus lavis*, and the CHO system. The baculovirus expression system has been proved to be a better system, and a number of foreign proteins have been well expressed. Recombinant proteins are soluble, with antigenicities, immunogenicities, and functional properties similar to those of their native counterparts. Baculovirus-infected insect cells perform many of the posttranslational modifications most higher eukaryotes do, which makes this expression system a valuable tool for production of biologically active proteins. Maeda *et al.* first used BmNPV to express the human α-interferon gene in *B. mori* cells and silkworm larvae [10]. The expression level in larvae hemolymph is very high (50 μg/mL). In this study, the novel high-efficient transfer vector pBn96, constructed according to the recent research on BmNPV gene regulation, was used in the expression of hBChE. We found that nhBChE could be expressed successfully in recombinant baculovirus-infected BmN cells and silkworm larvae. The level of rhBChE expression far exceeded that produced in any other expression system. We obtained as much as 35 mg/L of rhBChE in hemolymph, about 280-fold more than in CHO mammalian cells (125 μg/L). This is the first report of human BChE expression in silkworm with the highest level of yield so far. The expression can easily be amplified from cell culture to large-scale production in silkworm larvae. Alongside the perfectioning of techniques of artificial fodder and automatic silkworm raising, it will be an economical way to produce rhBChE, especially in a country with a long-standing history of silkworm raising such as China.

The results showed that rhBChE was highly similar to nhBChE in respect to substrate affinity, inhibitor sensitivity, and reactivation efficiency of the inhibited enzyme and

TABLE 1. Reactivation of sarin-inhibited BChE by HI-6

Reactivation time/hr	nmol BTCh hydrolyzed/30 min			% Reactivation	
	E	EI	EIR	HI-6	Spontaneous
rhBChE					
0.5	36.1 ± 0.3	1.1 ± 0.1	5.7 ± 0.3	13	1
2	36.1 ± 0.3	1.1 ± 0.1	24.2 ± 0.3	66	1
4	36.1 ± 0.3	1.1 ± 0.1	30.5 ± 0.4	84	2
nhBChE					
0.5	33.9 ± 0.2	1.2 ± 0.1	5.1 ± 0.2	12	1
2	33.9 ± 0.2	1.2 ± 0.1	22.5 ± 0.3	65	2
4	33.9 ± 0.2	1.2 ± 0.1	29.3 ± 0.5	86	2

E: normal BChE activity; EI: inhibited BChE activity; EIR: inhibited BChE activity reactivated by HI-6; BTCh, butyrylthiocholine. Values are means ± SD, N = 3 independent experiments in duplicate.

TABLE 2. BChE activity inhibited by polyclonal antibody

Dilution of antibody	Enzyme activity/nmol · min^{-1}		% Inhibition rate	
	nhBChE	rhBChE	nhBChE	rhBChE
1:100	1.12 ± 0.11	1.02 ± 0.10	7	10
1:50	1.01 ± 0.13	0.92 ± 0.11	16	19
1:10	0.91 ± 0.11	0.81 ± 0.11	24	28
1:5	0.88 ± 0.12	0.76 ± 0.12	27	34
1:1	0.72 ± 0.11	0.64 ± 0.10	40	43

nhBChE and rhBChE control activities: butyrylthiocholine 1.20 ± 0.10 and 1.13 ± 0.11 nmol · min^{-1}, respectively. Values are means ± SD, N = 3 independent experiments in duplicate.

immunoreactivity with antihuman BChE antibodies. Although rhBChE functions as well as nhBChE, rhBChE glycosylated at a relatively lower level.

We are grateful to Dr. Oksana Lockridge for the gift of pRc CMV–BChE plasmid. Our appreciation and thanks go to Professors De-Xu Zhu, Yu-Hui Zang, and Zhu Jie for their many constructive suggestions and help.

References

1. Mattes CE, Lynch TJ, Singh A, Bradley RM, Kellaris PA, Brady RO and Dretchen KL, Therapeutic use of butyrylcholinesterase for cocaine intoxication. *Toxicol Appl Pharmacol* **145:** 372–380, 1997.
2. Lockridge O, Genetic variants of human serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. *Pharmacol Ther* **47:** 35–60, 1990.
3. Allon N, Raveh L, Gilat E, Cohen E, Grunwald J and Ashani Y, Prophylaxis against soman inhalation toxicity in guinea pigs by pretreatment alone with human serum butyrylcholinesterase. *Toxicol Sci* **43:** 121–128, 1998.
4. Raveh L, Grauer E, Grunwald J, Cohen E and Ashani Y, The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol Appl Pharmacol* **145:** 43–53, 1997.
5. Masson P, Expression and refolding of functional human BChE from *E. Coli*. In: *Multiple Approaches to Cholinesterase* Functions (Eds. Shafferman A and Velan B), pp. 49–52. Plenum, New York, 1990.
6. Soreq H, Seidman S, Dreyfus PA, Zevin-Sonkin D and Zakut H, Expression and tissue-specific assembly of human butyrylcholine esterase in microinjected *Xenopus laevis* oocytes. *J Biol Chem* **264:** 10608–10613, 1989.
7. Lockridge O, Human cholinesterase gene expression in mammalian cells. *AD Report A* 227610.MF, USA, 1990.
8. Wang L, Qin J and Shen B, Expression of human stem cell factor in the baculovirus expression system. *Biochem Mol Biol Int* **37:** 729–736, 1995.
9. Karnoviky MJ and Root LA, A "direct-coloring" thiocholine method for cholinesterases. *J Histochem Cytochem* **12:** 219–221, 1964.
10. Maeda S, Kawai and Obinata T, Production of human α-interferon in silkworm using a baculovirus vector. *Nature* **315:** 592–594, 1985.