A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Characterization of molecular and enzymatic properties of three cholinesterases from the common bed bug, *Cimex lectularius*

빈대의 세가지 콜린에스터라제의 분자형태 및 효소학적 특성 연구

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February, 2014

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By

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Abstract

Acetylcholinesterase (AChE, EC 3.1.1.7) is a crucial enzyme in the insect nervous system as the potential main target sites for OPs and CBs. In most insect species, there are two different types of AChE which is encoded from two different loci of AChE gene. The common bed bug, *Cimex lectularius*, is synanthropic and attack humans by feeding blood. The bed bugs have re-emerged and distribute in the Europe, Australia, Canada and United states by increasing their numbers. It has unique feature which is three genes encoding different Cholinesterase (ChE) types (AChE1, AChE2 and Salivary gland specific cholinesterase (SChE)). In this study, I examined the molecular and enzyme properties of three cholinesterases (ChEs; ClAChE1, ClAChE2 and ClSChE) from the common bed bug, *Cimex lectularius*. As determined by activity staining and Western blotting after Native polyacrylamide gel electrophoresis, ClAChE1 was

the catalytically main enzyme and abundantly expressed in various tissues whereas ClAChE2 existed in central nervous system (CNS). Both ClAChEs existed in dimeric form connected by a disulfide bridge and were attached to the membrane via a glycophosphatidylinisitol-anchor. To investigate enzymatic properties, three ChEs were functionally expressed using baculovirus expression system. Kinetic analysis using *in vitro* expressed ClAChEs demonstrated that ClAChE1 had higher catalytic efficiency toward acetylcholine, supporting that ClAChE1 plays a major role in postsynaptic transmission. ClAChE2 showed higher catalytic efficiency toward butyrylcholine, wider substrate spectrum and selective inhibition by iso-OMPA. Inhibition assay using *in vitro* expressed ClAChEs revealed that ClAChE1 was generally more sensitive to insecticides. The relatively higher correlation between *in vitro* ClAChE1 inhibition and *in vivo* toxicity suggests that ClAChE1 is the more relevant toxicological target for organophosphate and carbamate insecticides.

ClSChE existed in salivary gland had negligible activity to hydrolyze acetylcholine, however ClSChE function is unrevealed. Under native conditions, ClSChE existed as soluble dimeric form connected by a disulphide bridge or soluble monomeric form. As an Immunohistochemistry of the salivary gland and salivary ducts, it existed as soluble form expressed in cell and passes through lumen. During sucking blood, I confirmed that the bed bug secreted ClSChE

through salivary canal in proboscis. In the result of acetylcholine/choline assay, ClSChE has a weak catalytic activity to hydrolyze acetylcholine. Based on the studies, ClSChE has non-neuronal function related to bloodsucking as acetylcholinesterase. These findings are useful in expanding our knowledge on insect AChEs and their evolution.

Key words: *Cimex lectularius,* molecular form, acetlycholinesterase, Baculovirus expression, Kinetics, Organophosphate, Carbamate, Salivary gland cholinesterase, Immunohistochemistry.

Abbriviations: ACh, acetylcholine; AChE, acetylcholinesterase; ATChI, acetylthiocholine iodide; BChE, butyrylcholinesterase; BTChI, butyrylthiocholine iodide; ClAChE1, *C. lectularius* AChE1 type of acetylcholinesterase; ClAChE2, *C. lectularius* AChE2 type of acetylcholinesterase; GPI, glycophosphatidylinositol; PIPLC, phosphatidylinositol-specific phospholipase C ; OP, organophosphate; CB, carbamate

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Abstract

I examined the molecular and enzyme properties of two acetylcholinesterases (AChEs; ClAChE1 and ClAChE2) from the common bed bug, *Cimex lectularius*. Native polyacrylamide gel electrophoresis followed by activity staining and Western blotting revealed that ClAChE1 was the catalytically main enzyme and abundantly expressed in various tissues. Both ClAChEs existed in dimeric form connected by a disulfide bridge and were attached to the membrane via a glycophosphatidylinisitol-anchor. Kinetic analysis using *in vitro* expressed ClAChEs demonstrated that ClAChE1 had higher catalytic efficiency toward acetylcholine, supporting that ClAChE1 plays a major role in postsynaptic transmission. Inhibition assay revealed that ClAChE1 was generally more sensitive to insecticides. The relatively higher correlation between *in vitro* ClAChE1 inhibition and *in vivo* toxicity suggests that ClAChE1 is the more relevant toxicological target for organophosphate and carbamate insecticides. ClAChE2 showed higher catalytic efficiency toward butyrylcholine, wider substrate spectrum and selective inhibition by iso-OMPA, which strongly suggested that ClAChE2 possesses similar properties of butyrylcholinesterase.

Although the physiological function of ClAChE2 remains to be elucidated, ClAChE2 may not yet be specialized to gain distinct non-neuronal functions as judged by its tissue distribution, molecular and kinetic properties. Our findings are useful in expanding our knowledge on insect AChEs and their evolution.

1 Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) terminates the synaptic transmission by hydrolyzing the neurotransmitter acetylcholine (Ach) into choline and acetic acid in the cholinergic nervous system of both vertebrates and invertebrates (Massoulie, Pezzementi et al. 1993). In the evolution of cholinesterases (ChEs), true ChEs with highly selective substrate specificity appear in the early bilaterians (Pezzementi and Chatonnet 2010). Two ChE genes, encoding AChE and butyrylcholinesterase (BChE), are usually observed in most lineages of vertebrates, whereas duplications of the terminates the synaptic transmission by hydrolyzing the neurotransmitter acetylcholine (ACh) into choline and acetic acid in the cholinergic nervous system of both vertebrates and invertebrates (Massoulie, Pezzementi et al. 1993). AChE is a key enzyme in the insect nervous system, in which the cholinergic system is essential (Fournier, Mutero et al. 1992), and is a

target for the action of organophosphate (OP) and carbamate (CB) insecticides. Structural alteration of AChE based on the substitution of a variety of amino acids results in insensitive enzyme which is one of the major mechanisms of OP and CB resistance (Fournier and Mutero 1994).*ace* gene, encoding AChE, are present in a few lineages, including nematodes, arachnids and insects. More than three *ace* loci have been cloned in nematodes and arachnids (Pezzementi and Chatonnet 2010), while two different *ace* loci have been identified in various insect species, such as *Aphis gossypii* (Li and Han 2002), *Schizaphis graminum* (Gao, Kambhampati et al. 2002), *Plutella xylostella* (Baek, Kim et al. 2005), *Blatella germanica* (Kim, Jung et al. 2006; Kim, Choi et al. 2010) and *Apis mellifera* (Kim, Cha et al. 2012). The first type of AChE, which is orthologous to *Drosophila ace* (*ace2*, encoding AChE2), is common to all insect species, while the second type of AChE is paralogous *to Drosophila ace* (*ace1*, encoding AChE1) and has not been identified in Cyclorrhaphan flies (Huchard, Martinez et al. 2006). It has been suggested that the two *ace* genes were derived from a duplication that occurred long before the differentiation of insects, whereas the *ace1* copy was lost in Cyclorrhapha during the course of evolution (Weill, Fort et al. 2002).

Following duplication, functional specialization of two AChEs appears to have occurred by gaining non-classical (non-neuronal) functions other than synaptic transmission. In *Culicidae* mosquitoes, AChE1 was proposed to have the main

synaptic function, whereas AChE2 to play a limited synaptic role and/or other non-synaptic roles (Weill, Fort et al. 2002; Huchard, Martinez et al. 2006). Likewise, AChE1 plays a major role in synaptic transmission whereas AChE2 has a limited neuronal function and/or other functions in *B. germanica* (Revuelta, Piulachs et al. 2009). Furthermore, knockdown of AChE1 resulted in 100% mortality and increased susceptibility to OP insecticides in *Tribolium castaneum*, suggesting the neuronal function of AChE1 (Lu, Park et al. 2012). Taken together, AChE1 has been generally considered as the major AChE involved in synaptic transmission in insects possessing both AChE1 and AChE2 based on its higher expression level (Baek, Kim et al. 2005; Kim, Jung et al. 2006; Lee, Kim et al. 2006; Lee, Kasai et al. 2007; Seong, Kim et al. 2012) and its association with insecticide resistance (Nabeshima, Mori et al. 2004; Weill, Malcolm et al. 2004; Kim, Choi et al. 2010; Lu, Park et al. 2012; Seong, Kim et al. 2012). In a recent study on honey bee AChE, however, AChE2 was determined to have neuronal functions unlike most other insects, with AChE1 having non-neuronal functions, including chemical defense (Kim, Cha et al. 2012). A more recent survey on the distribution of catalytically active AChE across a wide range of insect orders revealed that AChE2 is also employed as the main catalytic enzyme in a variety of insect species (Kim and Lee 2013), indicating frequent occasions of the *de novo* specialization of AChE2 as the main catalytic enzyme or the functional

replacement of AChE1 with AChE2.

In addition to the functionally distinct AChEs encoded from different *ace* loci, multiple molecular forms of each AChE can contribute to the functional diversification of AChE. Several structurally distinct forms of AChE have been identified in both vertebrates and invertebrates (Massoulie and Bon 1982; Fournier, Cuany et al. 1987; Gnagey, Forte et al. 1987; Perrier, Cousin et al. 2000; Badiou, Brunet et al. 2007; Kim, Choi et al. 2010). A number of studies reported three different molecular forms, such as amphiphilic dimer associated with cell membrane via a glycophosphatidylinositol (GPI)-anchor, hydrophilic water-soluble dimer and monomer (Fournier, Cuany et al. 1987; Gnagey, Forte et al. 1987; Kim, Choi et al. 2010; Kim, Cha et al. 2012). Furthermore, soluble dimer and monomer having faint AChE activity have been predominantly observed in non-neuronal tissues in the honey bee (Kim, Cha et al. 2012) and *Drosophila* (Kim and Lee 2013), respectively, and have non-neuronal functions.

The common bed bug *Cimex lectularius* L. (Hemiptera: Cimicidae) is an ectoparasitic pest feeding on the blood of humans and other mammals throughout entire life stages (Reinhardt and Siva-Jothy 2007) and is widely distributed in temperate and subtropical regions. Recently, two different *ace* loci encoding AChE1 (ClAChE1) and AChE2 (ClAChE2) were identified (Seong, Kim et al. 2012). Interestingly, *C. lecturarius* has one more ChE locus encoding a salivary

gland-specific ChE (ClSChE), which appears to have been duplicated from the common ancestor of *ace1* (Seong, Kim et al. 2012). Therefore, *C. lecturarius* is a unique model insect to study the evolution and functional diversification of different ChEs following sequential duplications. Since the ClSChE appears to have gained non-neuronal functions with a negligible level of AChE activity whereas ClAChE1 and ClAChE2 retained high catalytic activity, it is worth to evaluate and compare the biochemical properties of these two AChEs, thereby inferring their physiological functions and evolutionary status. In this study, the molecular forms and tissue distribution patterns of two ClAChEs were examined using ClAChE1- and ClAChE2-specific antibodies. In addition, two ClAChEs were expressed in Sf9 cells using a baculovirus expression system to determine their kinetic and inhibitory properties. Based on the catalytic properties and molecular nature, the physiological functions of each ClAChE were predicted and the evolutionary status of two ClAChEs was discussed. Our study would provide with more insight into the functional evolution of insect AChEs.

2 Materials and methods

2.1 Insect and Chemical

C. lectularius populations were maintained using an artificial feeding system

in the laboratory conditions as previously described (Yoon, Kwon et al. 2008; Seong, Kim et al. 2012). All chemicals were purchased from Sigma-Aldrich Co. (St Louis, Mo, USA), while OP and CB insecticides were obtained from Chem Service (West Chester, PA, USA).

2.2 Antibody

Rabbit anti-ClAChE1 polyclonal antibody was produced by Ab Frontier (Seoul, Korea). The two bovine serum albumin-fused peptide fragments [QDDPLVIDTKKGKVRGIT (position 109–26) and WLNPDDPIRNRDALDKM (position 488–504) for anti-ClAChE1] were synthesized. Rabbits were immunized three times with 0.5 mg of the two synthesized peptides. The Serum-specific antibodies were affinity-purified on columns using immobilized antigen peptides. The titer of the anti-ClAChE1 polyclonal antibody was approximately 2 mg/ml. In case of ClAChE2, the antibody was present from Kim et al (Kim and Lee 2013).

2.3 Electrophoresis, activity staining and western blotting

Protein samples were extracted from six different tissues (brain+ganglia, salivary gland, head, thorax, abdomen, and leg) by following previously described (Kim, Cha et al. 2012). Protein samples were obtained from head, thorax, and abdomen without removal of CNS. Twenty micrograms of protein samples were

separated by 7.5 % native-PAGE gel for 100 min at 120 V. After native-PAGE, one set of gels was stained by AChE activity staining (Lewis and Shute 1966), and the other set was used for Western blotting as described by Kim et al (Kim, Cha et al. 2012). To determine molecular properties of ClAChE1 and ClAChE2, protein samples were treated with or without 40 mM of β-mercaptoethanol and 0.05 U of phospholipase C (PIPLC) for 5 min at 37°C. The migration patterns of the samples were investigated by SDS-PAGE and native-PAGE, respectively.

2.4 Generation of recombinant baculoviruses and Purification

The partial fragments of ClAChE1 and ClAChE2 with truncated C-terminal hydrophobic regions were amplified from respective clones (Seong, Kim et al. 2012) by Ex Taq (TAKARA, Japan) at 95°C for 2 min, (95°C for 30 s, 65°C for 30 s, 72°C for 2 min) x 35 cycles with gene-specific primers containing restriction enzyme sites and x6 His tag sequence as shown in Table 1. The amplified ClAChEs DNA fragments were digested with *Xba* I and *Kpn* I (Koschem, Korea) and inserted into pBacPAK8 (Clonetech, Palo Alto, CA, USA) that was then digested with the same restriction endonucleases. Recombinant baculoviruses expressing ClAChE1 and ClAChE2 in SF9 cells were generated as described (Kim, Choi et al. 2010). Virus-infected cells were incubated for 84 h at 27°C. The expressed ClAChEs were collected from supernatants by centrifugation at 5,000 g

for 15 min, and concentrated by ultracentrifugation at 35,000 rpm for 15 min at 4°C in an ULTRA 4.0 ultracentrifuge (Hanil Science Industrial, Korea). Then ClAChE1 and ClAChE2 were purified by the Fast Protein Liquid Chromatography (FPLC) system from supernatants concentrated by Ultra Amicon YM-30 (Millipore, Bedford, MA, USA) following some modified methods as described (Kwon, Choi et al. 2012). Approximately 3-4 ml of concentrated supernatant was loaded into a 5-ml HisTrap HP affinity column (Amersham Biosciences Corp., Piscataway, NJ) at a flow rate of 5 ml/min using an AKTA FPLC (Amersham Bioscience UPC-900). Purified proteins were eluted with 250 ml of imidazole and collected by Frac-920 fraction collector (Amersham Biosciences). The eluted protein was concentrated with the YM-50 filter and exchanged its buffer condition to 0.1 M Tris buffer containing 0.02M NaCl and 20% glycerol (pH 7.8). The samples were stored at - 80°C until use.

2.5 Kinetic assay

The enzyme assay of expressed ClAChEs was conducted with acetylthiocholine iodide (ATChI) and butyrylthiocholine iodide (BTChI) at eight

Table 1. The primers used for in vitro expression of ClAChE1 and ClAChE2 by baculovirus expression system

Primers	**Sequence (5'→3')***
5BAC_Clace1	AGAGTCTAGAATGAGGTGGCTGTTAGTGGCTA
3BAC_Clace1(not GPI)	CCCGGTACCATGATGATGATGATGATGGTGGCATTCCTGTACTGGATTTG
5BAC_Clace2	AGAGTCTAGAATGTCTCCTTGGATTGGGTGCGT
3BAC_Clace2(not GPI)	CCCGGTACCATGATGATGATGATGATGGTGGTTCTGTACTAATTCTACATC

* Red letter indicates Xba I and Kpn I sequences for respective forward and reverse primers. Green letters show 6 x his tag coding sequence.

different concentrations (0.05 to 1 mM) according to a previously described method with some modifications (Ellman, Courtney et al. 1961; Grant, Bender et al. 1989). For enzyme kinetics, 4 ng of purified ClAChEs was added to each well containing 95 µl substrate mixture with 0.4 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction was allowed to proceed at 30°C, and the kinetic constants were measured at 412 nm for a period of 5 min at 20-s intervals using a VERSAmax microplate reader (Molecular Devices, Menlo Park, CA, USA). The K_m and V_{max} values for each substrate were calculated from a Lineweaver-Burk plot (SigmaPlot ver. 9.0, SYSTAT Software Inc., Chicago, IL, USA).

2.6 In vitro inhibition assay

The inhibition of ClAChEs was assayed at different concentrations (10^{-9} to 10^{-1} mM) of each of three cholinesterase-specific inhibitors, such as 1,5-bis(4-allyldimethylammoniumpheyl)pentan-3-one dibromide (BW284C51), eserine and tetra (monoisopropyl) pyrophosphortetramide (Iso-OMPA), four OPs [chlorpyrifos-methyl-oxon, malaoxon, paraoxon, and dichlorvos (DDVP)], and two CBs (carbaryl and carbofuran) according to a previously described method with some modifications (Zhu and Brindley 1992). In brief, each inhibitor stock solution was prepared in acetone, serially diluted in acetone to test concentrations, and mixed with the substrate reaction mixture, in which final acetone

concentration was 0.1%. Each expressed ClAChE (10 ng) was added to the reaction mixture containing 1 mM ATChI, 0.4 mM DTNB, and various concentrations of inhibitors (10^{-8} to 10^{-3} mM for BW284C51 and eserine; 10^{-6} to 10^{-1} mM for Iso-OMPA; 10^{-9} to 10^{-2} mM for OPs; and 10^{-9} to 10^{-1} mM for CBs in final), and then the activity was measured at 20-s intervals for 5 min at 30°C using a microplate reader as described previously. The median inhibition concentration (IC_{50}) for each inhibitor was determined based on the log-concentration vs. probit (% inhibition) regression analysis using Microsoft Office Excel. The entire inhibition assay was carried out in three replicates.

2.7 In vivo bio assay by topical application

To determine the sensitivity of the bed bug against insecticides, 4^{th} instars were treated with various concentrations of four OPs (chlorpyrifos-methyl, malathion, parathion, and DDVP) and two CBs (carbaryl and carbofuran) dissolved in acetone. A 0.2-µl droplet of the insecticide solution was applied to the surface of the abdomen with a repeating dispenser (Hamilton Co., Reno, NV, USA). Ten bed bugs per dose were used in each assay. All of the bioassay were performed at 27±1°C and repeated three times on different days. The median lethal dose (LD_{50}) value was determined by Probit analysis (SPSS).

3 Results

3.1 Tissue distribution patterns of ClAChE1 and ClAChE2

To determine the tissue distribution patterns of two ClAChEs, proteins extracted from six different tissues (brain + ganglia, salivary gland, head, thorax, abdomen and leg) were separated on the native-PAGE gel and their migration patterns were visualized by AChE activity staining and Western blotting (Fig. 1). The high AChE activities were investigated not only in the tissues mostly composed of the central nervous system (CNS), such as brain and ganglia, but also in the other tissues containing CNS (head, thorax and abdomen). Interestingly, AChE activity was also detected in the peripheral nervous system (PNS), such as leg (Fig. 1B). As indicated by a comparison of AChE activity staining and Western blotting, ClAChE1 (band ‘a’) showed much higher enzyme activity than ClAChE2 (band ‘b) in all tissues. In addition to the main AChE band showing catalytic activity (band ‘a’), the fast migrating bands of ClAChE1 (band ‘c’) were observed with weak enzymatic activities (Fig. 1). Western blot analysis revealed that ClAChE1 was abundantly expressed in all tissues (Fig. 1A), whereas ClAChE2 was generally expressed in the CNS (Fig. 1C).

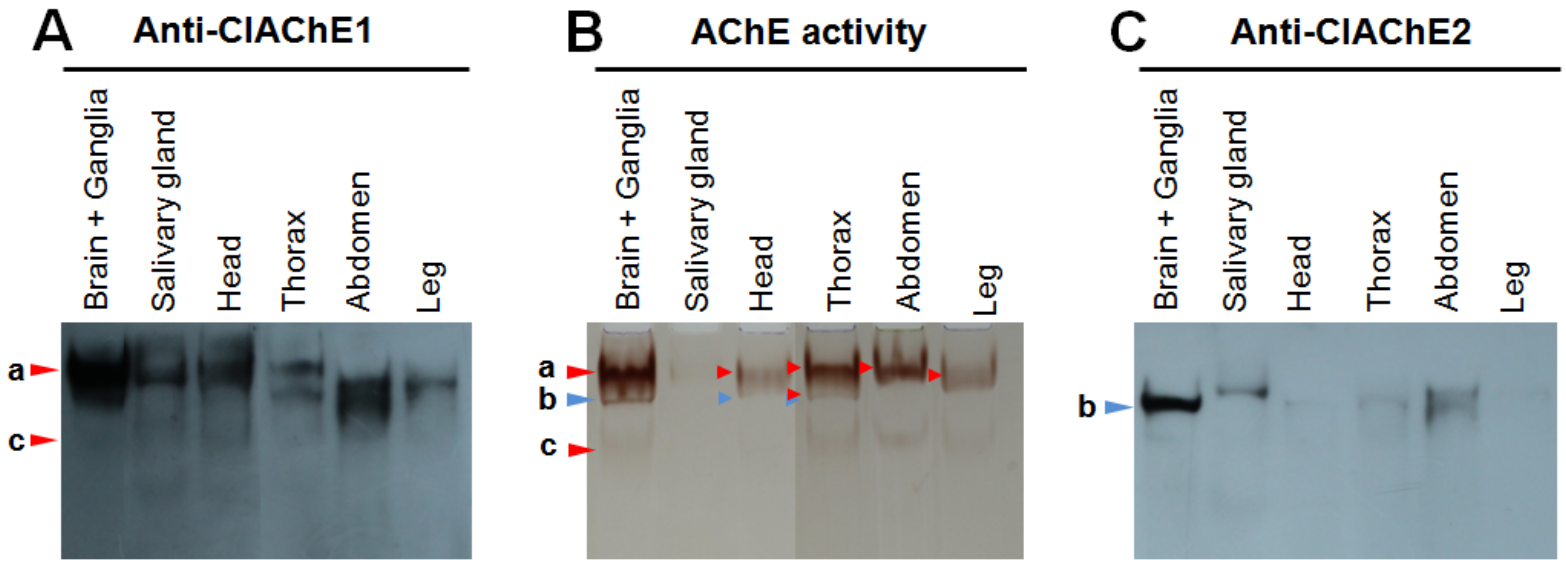

Figure 1. Tissue distribution of ClAChE1 and ClAChE2 as assembled by native-PAGE and Western blot analysis. After electrophoresis, one gel was stained for AChE activity (B). Other gels were analyzed by Western blotting using anti-ClAChE1 (A) and anti-ClAChE2 (C) polyclonal antibody.

3.2 Molecular forms of ClAChE1 and ClAChE2

To determine soluble nature of ClAChE1 and ClAChE2, crude protein was extracted from the brain and ganglia with 0.1 M Tris-HCl buffer in the presence or absence of Triton X-100. In the presence of Triton X-100, the putative dimeric forms of both ClAChE1 (130 kDa, band a) and ClAChE2 (140 kDa, band b) were strongly detected (Fig. 2A, see the Triton X-100 (+) lanes). When protein samples were extracted with the buffer without Triton X-100, however, the amounts of the dimeric form (band a) of ClAChEs considerably increased with the monomeric form (band c) being slightly increased (Fig. 2A. see the Triton X-100 (-) lanes), suggesting that soluble dimeric and monomeric forms of ClAChE1 are present as well. In case of ClAChE2, the dimeric form (band b) was barely detected in the absence of Triton X-100, which indicates that ClAChE2 mostly exists as a membrane-anchored dimer.

To confirm the membrane-anchored properties, protein samples extracted from the brain and ganglia with the Triton X-100-containing buffer were treated with phosphatidylinositol-specific phospholipase C (PIPLC). Amphiphilic form of ClAChE1 (band a) and ClAChE2 (band b) were converted to the hydrophilic forms (band d and band e for ClAChE1 and ClAChE2, respectively) (Fig. 2B, see the PIPLC (+) lanes), indicating that both ClAChEs are associated with cell membrane via a GPI-anchor.

The molecular weights (MWs) of ClAChEs were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with or without β-mercaptoethanol reduction. Under non-reduction conditions, bands of approximately 130 kDa (band a) and 140 kDa (band b) were strongly visualized by ClAChE1- and ChAChE2-specific antibodies (Fig. 2C, see the Reduction (-) lanes). The dimeric forms of ClAChEs were completely converted to monomeric forms of 67 kDa (band c' for ClAChE1) and 72 kDa (band f for ClAChE2) under reduction conditions, supporting the presence of a disulfide bond in the dimer conformations.

In summary, as depicted in Fig. 3, ClAChE1 appears to exist as the GPI-anchored dimer as the major form with the soluble dimer or monomer as the minor form. In contrast, ClAChE2 appears to be present solely as the GPI-anchored dimer.

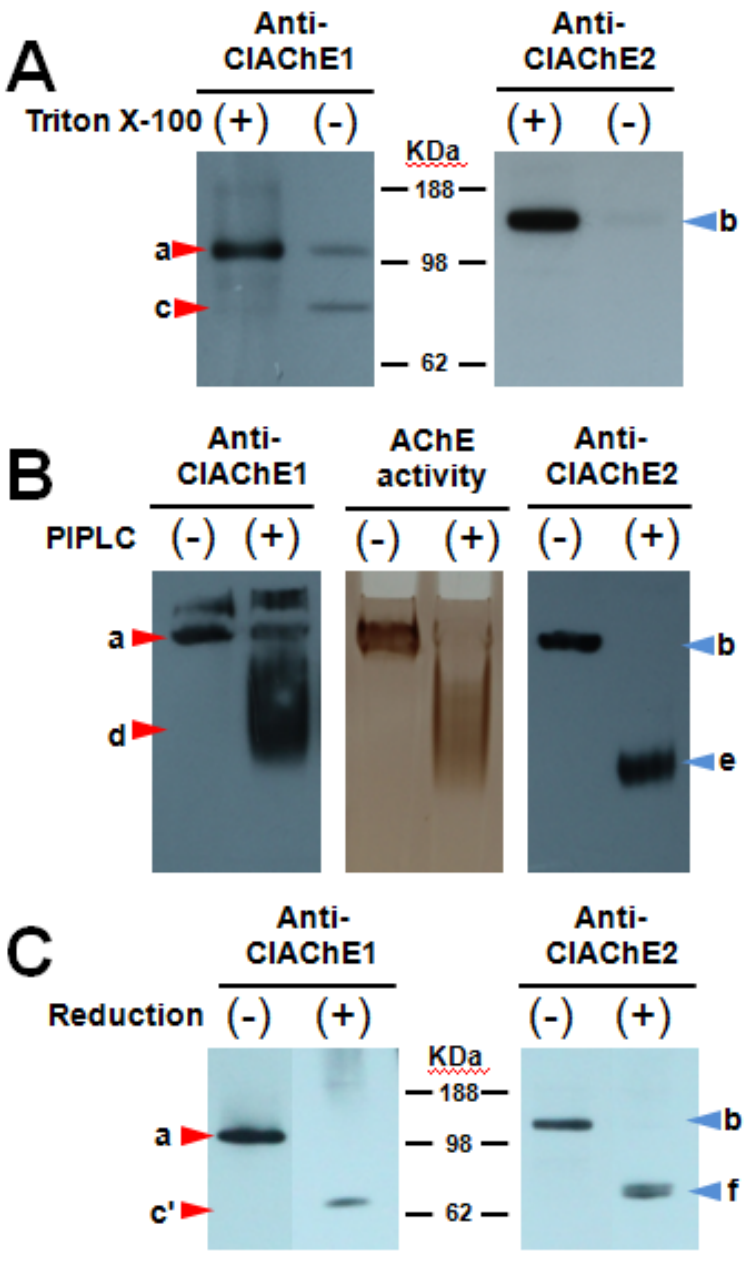
A
Anti-
ClAChE1
Anti-
ClAChE2
Triton X-100 (+) (-)
(+) (-)
KDa
188
98
62
a
c
b
B
Anti-
ClAChE1
AChE
activity
Anti-
ClAChE2
PIPLC (-) (+)
(-) (+)
(-) (+)
a
d
b
e
C
Anti-
ClAChE1
Anti-
ClAChE2
Reduction (-) (+)
(-) (+)
KDa
188
98
62
a
c'
b
f

Figure 2. Molecular characterization of ClAChE1 and ClAChE2 by native-PAGE and Western blot analysis. To investigate the soluble nature of two ClAChEs, protein samples were extracted from the brain and ganglia of bed bug with 0.1 M Tris-HCl buffer in the presence or absence of 0.5% Triton X-100 (A). Proteins samples were treated with PIPLC to determine the GPI-anchor properties of ClAChEs (B). To determine the multimer formation of two ClAChEs, proteins were separated under reduction or non-reduction condition using β-mercaptoethanol (C).

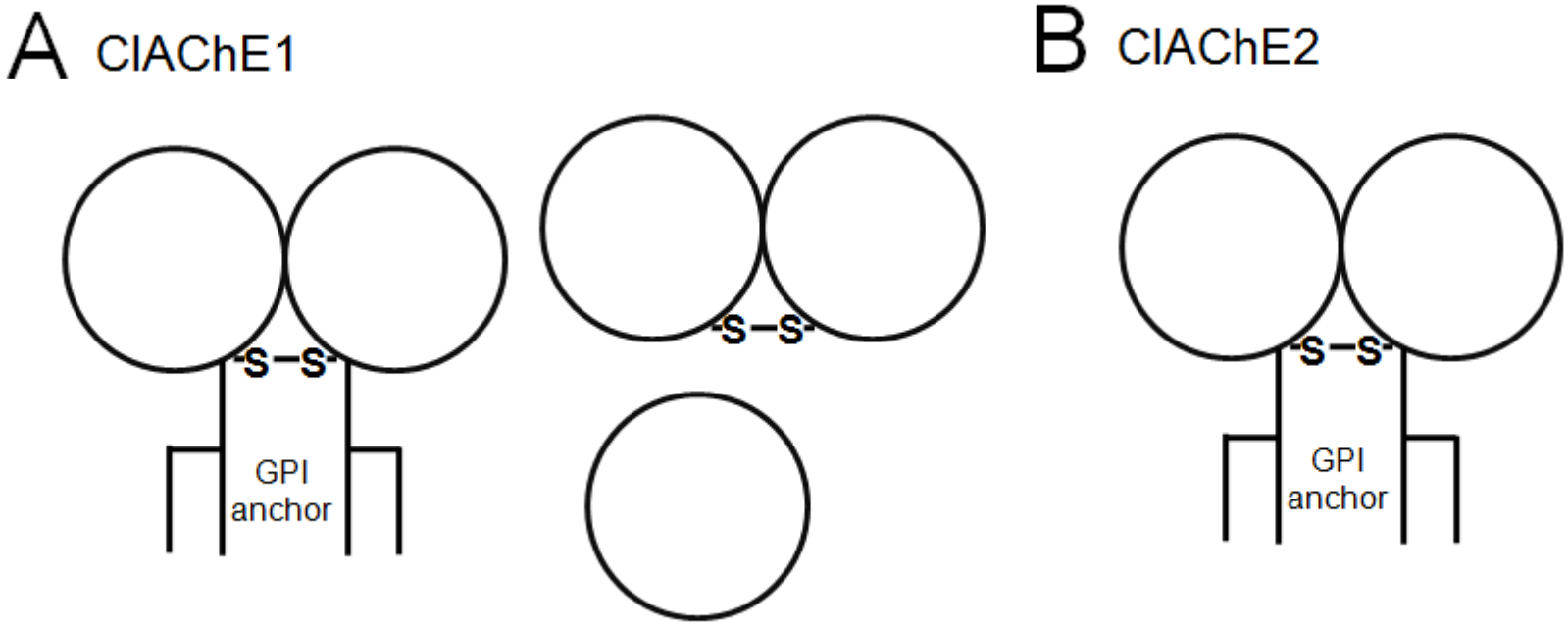

Figure 3. Schematic diagrams of the molecular structures of ClAChE1 (A) and ClAChE2 (B). GPI-anchored dimer of ClAChE1 and ClAChE2 are predominately expressed, while soluble dimer and monomer of ClAChE1 were also investigated.

3.3 In vitro expression and purification of recombinant ClAChEs

Both ClAChE1 and ClAChE2 were successfully expressed by a recombinant baculovirus expression system as judged by AChE activity staining and Western blotting with the anti-His tag antibody (data not shown). The *in vitro* expressed recombinant ClAChEs were purified sequentially and their activities were confirmed by SDS-PAGE (data not shown). The overall purification factors for ClAChE1 and ClAChE2 were 45- and 21-fold, respectively, as summarized in Table 2. Approximately 40 μg of ClAChE1 and 30 μg of ClAChE2 were obtained from 20 mg of cell culture supernatants with a specific activity of 291 and 19 μmol/min/μg protein, respectively.

Table 2. Purification of *in vitro* expressed ClAChE1 and ClAChE2 from Sf9 cell cultured supernatant by concentration with 30 kDa cutoff filter, ultra centrifugation and his-tag affinity chromatography.

Sample	Step	Volume (ml)	Total activity (mM/min)	Total protein (mg)	Specific activity (μM/min/μg protein)	Yield (%)	Purification factor (fold)
ClAChE 1	Cell cultured supernatant	70	122	18.9	6.48	100	
	Filter concentration (30 kDa cutoff)	3.8	123	14.7	8.37	77.8	1.29
	Ultra centrifuge	3.5	99.6	12.1	8.25	63.9	1.27
	His-tag affinity column	0.25	11.7	0.04	291	0.21	44.9
ClAChE 2	Cell cultured supernatant	70	20.0	21.7	0.92	100	
	Filter concentration (30 kDa cutoff)	3.8	20.5	20.6	1.00	95.1	1.08
	Ultra centrifuge	3.6	18.4	13.0	1.41	59.9	1.54
	His-tag affinity column	0.25	0.63	0.03	19.3	0.15	21.0

3.4 Catalytic properties of ClAChEs

The catalytic properties of ClAChEs were determined using two ChE substrates, acetylthiocholine iodie (ATChI) and butyrylthiocholine iodide (BTChI). The kinetic parameters, including K_m and V_{max} values, of ClAChE1 and ClAChE2 for the two substrates are presented in Table 3. In a cross-enzyme comparison of ClAChE1 and ClAChE2, ClAChE1 exhibited 22- and 6-fold higher hydrolyzing efficiencies than ClAChE2 toward ATChI and BTChI, respectively, as indicated by V_{max} values. Both ClAChEs showed similar K_m values for ATChI, while K_m value of ClAChE2 was lower than that of ClAChE1 toward BTChI, demonstrating that ClAChE2 has a 12-fold higher substrate specificity to BTChI than ClAChE1. As judged by V_{max}/K_m values, ClAChE1 showed a 22-fold higher catalytic efficiency toward ATChI than ClAChE2, while ClAChE2 exhibited a 1.3-fold higher catalytic efficiency than ClAChE1 for BTChI. The substrate specificities of ClAChE1 and ClAChE2 were determined to be 0.43 and 1.46, respectively, as indicated by the ratio of V_{max}(BTChI)/V_{max}(ATChI), further suggesting that ClAChE2 has a 3.4-fold wider substrate spectrum compared to ClAChE1. In summary, these results show that ClAChE1 has a higher substrate specificity and a higher catalytic efficiency toward ATChI, whereas ClAChE2 exhibited a wider substrate spectrum and a higher catalytic efficiency toward BTChI.

Table 3. Substrate kinetic properties of the ClAChE 1 and ClAChE2 that were *in vitro* expressed using baculovirus

Substrate	Kinetic properties	ClAChE1	ClAChE2	Ratio [a]
ATChI	V_{max} (μmol/min/μg)	333 ± 167	15.5 ± 4.6	21. 5
	K_m (mM)	0.16 ± 0.1	0.16 ± 0.13	1
	V_{max}/K_m	1663	77.5	21. 5
BTChI	V_{max} (μmol/min/μg)	143	22.7 ± 3.72	6.29
	K_m (mM)	0.3	0.02 ± 0.02	11.9
	V_{max}/K_m	500	945	0.53
Substrate specificity	V_{max}(BTChI)/ V_{max}(ATChI)	0.43	1.46	0.29

[a] ClAChE1 / ClAChE2

3.5 Inhibitiory properties of ClAChEs

The inhibitory properties of the two ClAChEs were determined using various concentrations of three reversible cholinesterase inhibitors, four OPs, and two CBs (Table 4). Both ClAChEs were effectively inhibited by BW284C51 and eserine. However, Iso-OMPA, a BChE-specific inhibitor, inhibited ClAChE2 but not ClAChE1 at the highest concentration tested (0.1 mM), suggesting that ClAChE1 possesses the typical features of AChE, whereas ClAChE2 exhibits inhibitory properties similar to those of vertebrate BChE. As determined by the IC_{50} values, ClAChE2 was 25- and 12-fold more sensitive to BW284C51 and eserine, respectively, compared with ClAChE1. The comparison of the IC_{50} values of ClAChE1 and ClAChE2 against OPs revealed that ClAChE1 is more effectively inhibited by some OPs (5-, 10-, and 1.6-fold more inhibited by chlorpyrifos-oxon, DDVP, and paraoxon, respectively) than ClAChE2, whereas ClAChE2 is approximately 4,000-fold more sensitive to malaoxon than ClAChE1. In the inhibition test using CBs, different sensitivities were observed depending on the inhibitor structure. ClAChE2 was 9-fold more sensitive to carbaryl than ClAChE1, whereas ClAChE1 was 3-fold more sensitive to carbofuran compared with ClAChE2.

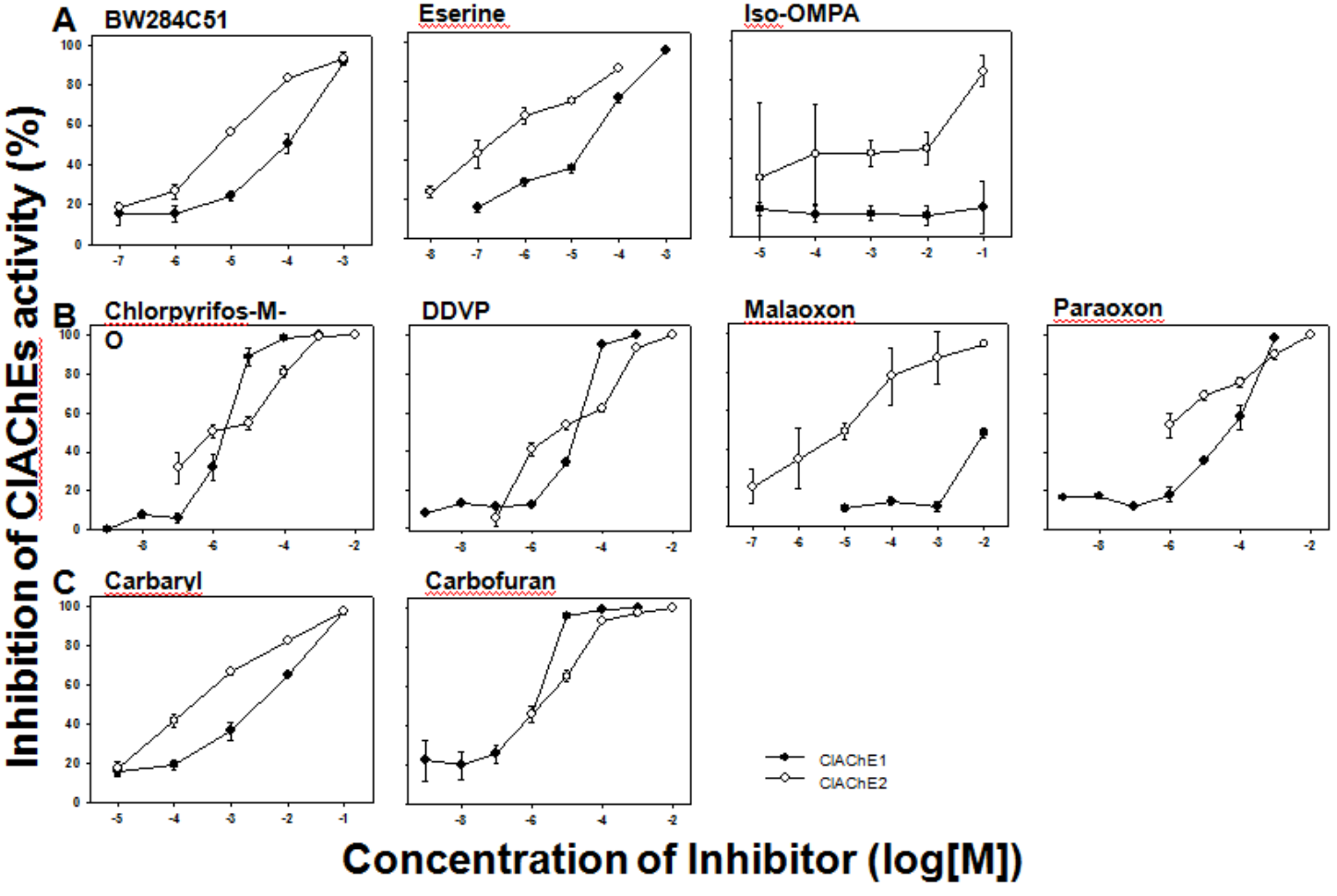
A
BW284C51
Eserine
Iso-OMPA
B
Chlorpyrifos-M-O
DDVP
Malaoxon
Paraoxon
C
Carbaryl
Carbofuran
ClAChE1
ClAChE2
Inhibition of ClAChEs activity (%)
Concentration of Inhibitor (log[M])

Figure 4. Inhibition of ClAChE1 (●) and ClAChE2 (○) by cholinesterase-specific inhibitors (A), OPs (B) and CBs (C). The results are the means of three determinations (n=3). Vertical bars indicated standard errors.

Table 4. IC_{50} (M) values of different inhibitors against the ClAChE1 and ClAChE2 that were *in vitro* expressed using baculovirus

Inhibitor	ClAChE1	ClAChE2	Ratio [a]
BW284C51	$(1.3 \pm 0.6) \times 10^{-4}$	$(5.1 \pm 0.65) \times 10^{-6}$	0.039
Eserine	$(2.33 \pm 0.58) \times 10^{-6}$	$(1.93 \pm 2.18) \times 10^{-7}$	0.083
Iso-OMPA	NI [b]	$(1.49 \pm 1.52) \times 10^{-3}$	-
Chlorpyrifos-M-O	$(1.82 \pm 0.41) \times 10^{-6}$	$(9.0 \pm 2.61) \times 10^{-6}$	4.94
DDVP	$(1.15 \pm 0.02) \times 10^{-6}$	$(1.22 \pm 0.3) \times 10^{-5}$	10.6
Malaoxon	$(1.0 \pm 0.1) \times 10^{-2}$	$(2.03 \pm 0.31) \times 10^{-6}$	2.03×10^{-4}
Paraoxon	$(5.29 \pm 2.24) \times 10^{-5}$	$(8.62 \pm 2.96) \times 10^{-5}$	1.63
Carbaryl	$(2.38 \pm 0.12) \times 10^{-3}$	$(2.66 \pm 1.24) \times 10^{-4}$	0.112
Carbofuran	$(6.25 \pm 0.53) \times 10^{-7}$	$(1.9 \pm 0.47) \times 10^{-6}$	3.04

[a] ClAChE2 / ClACHE1; [b] No inhibition at the highest concentration tested (0.1 mM)

3.6 In vivo toxicity of OPs and CBs and its correlation with in vitro AChE inhibition

To determine the toxicity of insecticides against bed bugs, various concentrations of four OPs and two CBs were topically applied to bed bugs (Table 5). When approximately 20 ng of OPs were administered to a bed bug, 100% moralities were observed with all of the OPs with the exception of malathion, suggesting that the bed bug is more sensitivity to chlorpyrifos, DDVP, and parathion than malathion, as indicated by the LD_{50} values. In the bioassay using CBs, carbofuran exhibited a 23-fold higher insecticidal activity against bed bugs than carbaryl.

When these LD_{50} values were correlated with the corresponding IC_{50} values for six different insecticides (Fig. 5), the level of *in vivo* toxicity exhibited a higher correlation with the level of *in vitro* ClAChE1 inhibition (r^2 = 0.7054) compared with that obtained with ClAChE2 (r^2 = 0.1881). This finding indicates that the toxicity of these insecticides is more affected by the inhibition of ClAChE1 than that of ClAChE2 in the bed bug.

Table 5. Median lethal doses (LD_{50}) of six insecticides to *Cimex lectularius*.

Insecticides	N	χ^2	df	Slope ± SE	LD_{50} (95% CL), ng/bed bug
Carbaryl	180	37.7	13	3.3 ± 0.2	42.2 (35.3 ~ 48.1)
Carbofuran	120	42.2	7	4.1 ± 0.2	1.6 (1.3 ~ 1.9)
Chlorpyrifos-metyhyl	180	27.2	13	5.7 ± 0.3	5.5 (5.1 ~ 5.8)
DDVP	150	86.5	13	3.7 ± 0.2	8.6 (7.6 ~ 10.2)
Malathion	180	72.4	13	3.0 ± 0.1	29.9 (22.8 ~31.8)
Parathion	180	77.6	13	4.4 ± 0.5	4.8 (4.1 ~ 5.5)

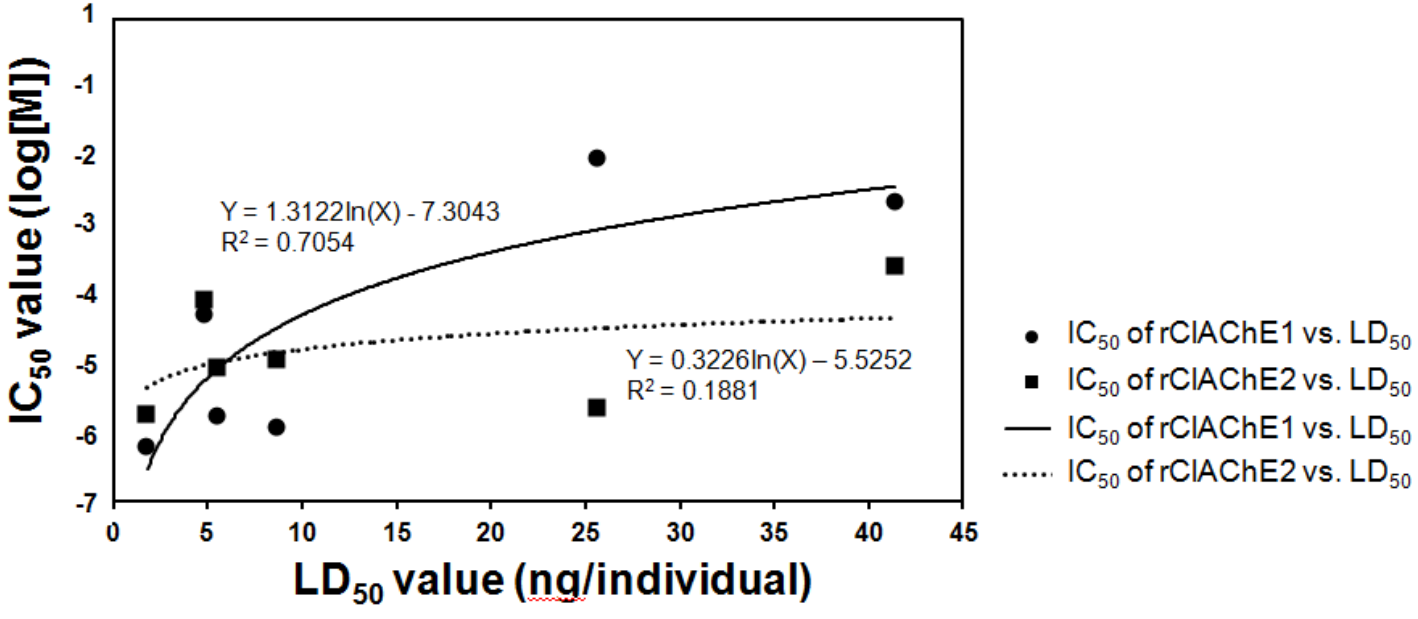

Figure 5. Correlation analysis of IC_{50} values of ClAChE1 (●) and ClAChE2 (■) with LD_{50} values of bed bug. The R2 value was assessed by log repression analysis (Microsoft excel 2010).

4 Discussion

4.1 Comparison of the properties of ClAChEs with other insect AChEs

In this study, I investigated the tissue distributions, molecular and kinetic properties of ClAChE1 and ClAChE2, which are summarized together with those of other invertebrate ChEs in Table 6. ClAChE1 is the major catalytic AChE accounting for most of *in vivo* activity, and widely distributed in both the CNS and PNS whereas ClAChE2 is found only in the CNS. According to Seong et al. (Seong, Kim et al. 2012), transcription level of ClAChE1 was approximately 5.5-fold higher than that of ClAChE2, supporting the fact that ClAChE1 is the major AChE. In fact, a comparison of the catalytic properties of *in vitro*-expressed ClAChE1 and ClAChE2 also confirmed that ClAChE1 is the catalytically more efficient than ClAChE2 (Table 3), further suggesting that AChE1 plays a more critical role in the synaptic transmission compared to ClAChE2 in the bed bug. In addition, ClAChE1 showed higher substrate specificity to ATChI compared to ClAChE2 (Tables 3 and 6). All other AChE1 examined (i.e., BgAChE1, BmAChE1 and AmAChE1) also showed similar tendency by having higher or similar substrate specificity to ATChI, suggesting that the AChE1 lineage may have retained such ACh-specificity during the evolution (Table 6). In case of *A. mellifera,* where the synaptic function of AChE1 was replaced with AChE2 (i.e., AmAChE2) unlike in most other insects, it is intreguing that the ACh-specificity

Table 6. Comparison of catalytic and molecular characteristics of different AChEs from *Cimax lectularius, Blatella germanica, Apis mellifera, Vestalis gracilis* and *Bursaphelenchus xylophilus*

Insect or nematode	AChE	*in vivo* activity	Relative substrate specificity (to ATChI)	Molecular form	Reference
Cimex lectularius	ClAChE 1	++	3.4	MA/S	Current study
	ClAChE 2	+	1	MA	
Blatella germanica	BgAChE 1	++	3.7	MA	Kim et al., 2010
	BgAChE 2	+	1	MA	
Apis mellifera	AmAChE 1	ND	0.98	S	Kim et al., 2012
	AmAChE 2	++	1	MA	
Vsstalis gracilis	VgAChE 1	++	-	MA	Kim et al., 2013
	VgAChE 2	++	-	MA/S	
Bursaphelenchus xylophilus	BxAChE 1	++	8.3	MA	Kang et al., 2011
	BxAChE 2	++	1	MA	
	BxAChE 3	ND	0.246	S	

[a] The symbol '++' indicates that the intensity of AChE activity stained band was much greater than the other AChE bands in the native polyacrylamide gel. The symbol '+' represents that the band was visualized by AChE activity staining, but weaker than the '++' AChE. 'ND' indicates that the activity was not detectable by AChE activity staining.

[b] Relative substrate specificity was calculated by dividing the value of V_{max}(ATChI)/ V_{max}(BTChI) of AChE1 with the value of V_{max}(ATChI)/ V_{max}(BTChI) of AChE2. The inverse values indicate the relative substrate specificity to BTChI. The symbol '-' indicates no data available.

[c] The abbreviations MA and S indicate the membrane-anchored form and soluble form, respectively.

of AmAChE2 is significantly increased to the level of AmAChE1 (Kim, Cha et al. 2012). Taken together, the ACh-sepecific hydrolysis may be a typical crierion for detetmining the major AChE having synaptic function in insects.

The analysis of the inhibition of ClAChEs by OPs and CBs revealed that no apparent tendency is present in the inhibition profile by the different inhibitors. In addition, cross-species comparison of the OP and CB inhibition between AChE1 and AChE2 did not deduce any specific inhibition patterns of AChE1 vs. AChE2 in the species examined (Table 7) (Kim, Choi et al. 2010; Lang, Zhang et al. 2010; Kim, Cha et al. 2012; Kim, Kwon et al. 2013; Kim, Kwon et al. 2013). For example, ClAChE1 was more sensitive to DDVP than ClAChE2 but AChE2 was more sensitive than AChE1 to DDVP in other insects examined. In cases of inhibition by paraoxon and carbaryl, no clear tendency was observed either in the inhibition profile between AChE1 and AChE2 in all the species examined (Table 7). Nevertheless, the level of *in vivo* toxicity to bed bugs was more highly correlated with the level of *in vitro* ClAChE1 inhibition compared with that obtained with ClAChE2, which implies that ClAChE1 is the more relevant target for these insecticides in the bed bug and further supports the notion that ClAChE1 is the main synaptic AChE. In the case of *A. mellifera*, where AmAChE2 is the main catalytic enzyme, it has also been suggested that the *in vivo* toxicity is more closely related with the *in vitro* inhibition of AmAChE2 (Kim, Cha et al. 2012).

Table 7. Comparison of inhibition ratio by organophosphate and carbamate insecticides between ClAChE1 and ClAChE2

Inhibitor	Inhibition ratio [a]				
	Cimex lectularius [b]	*Blattella germanica* [c]	*Apis melifera* [d]	*Vestalis gracilis* [e]	*Bombyx mandarina* [f]
DDVP	10.0	0.04	0.14	1.05×10^{-3}	0.29
Paraoxon	1.6	3.4	0.02	8.23	0.75
Carbaryl	0.1	0.04	2.86×10^{-4}	1.23	0.12

[a] IC_{50} of AChE2 / IC_{50} of AChE1
[b] Current study
[c] Kim et al ., 2010
[d] Kim et al., 2012
[e] Kim et al., 2013
[f] Lang et al., 2010

Interestingly, ClAChE2 was prominently more sensitive to malaoxon than ClAChE1, which implies that malathion toxicity to bed bugs is likely more attributed to the selective inhibition of ClAChE2.

The AChE insensitivity mechanism is known to be the major mechanism of resistance to OPs and CBs in many arthropod pests (Nabeshima, Mori et al. 2004; Weill, Malcolm et al. 2004). Structurally altered AChEs that are insensitive to OPs and CBs are generated by various mutations at several conserved positions in the AChE with the classical postsynaptic function. Except for the cyclorrhaphan flies that express only AChE2, several mutations conferring OP and CB resistance have been identified in AChE1 in most insects (Ramphul, Boase et al. 2009; Wu, Li et al. 2010). Determination of ClAChE1 as the toxicologically relevant AChE in the bed bug will facilitate the identification of germane mutations associated with AChE insensitivity, which would result in OP and CB resistance in this pest.

As shown in Fig. 2, the GPI-anchored form was the predominant molecular form of both ClAChE1 and ClAChE2. Without exception, major catalytic activity is associated with the membrane-anchored form of AChE, as observed in the bed bug (this study), German cockroach (Kim, Choi et al. 2010), honey bee (Kim, Cha et al. 2012), pinewood nematode (Kang, Lee et al. 2011) and damsel fly (Kim, Kwon et al. 2013) (Table 4). This finding suggests that the membrane-bound property is likely the prerequisite for the high catalytic efficiency required for a typical synaptic AChE. In contrast, all the soluble forms, including the ClSChE,

AmAChE1 and BxAChE3, have little or low catalytic activity (Table 6). Interestingly, these soluble ChEs are known to be abundantly expressed in non-neuronal tissues, suggesting that they have gained non-neuronal functions other than synaptic transmission (Kang, Lee et al. 2011). With this in mind, the presence of soluble form of ClAChE1 suggests that it may have evolved to gain non-neuronal function along with the ClSChE. Since both ClSChE and ClAChE1 appear to be paralogous, being duplicated from the common ancestor of *ace1* (Seong, Kim et al. 2012), they may have retained the traits for non-neuronal functions throughout the evolution.

Unlike ClAChE1, ClAChE2 is catalytically efficient toward BTChI, has a wider substrate spectrum and is selectively inhibited by iso-OMPA, all of which demonstrate that ClAChE2 resembles the vertebrate BuChE in its enzymatic properties. This BuChE-like properties of ClAChE2 was also observed in other invertebrate AChE2s (Table 6), including BgAChE2 (*B. germanica*) and BxAChE2 (*B. xylophilus*), suggesting that the BuChE-like properties of AChE2 is well conserved throughout the evolution. Interestingly, all insect/invertebrate AChE2s examined so far are CNS-specific, further implying that they may be involved in synaptic transmission, perhaps in the neurons different from those of AChE1 as reported in *B. germanica* (Kim, Jung et al. 2006). Since the vertebrate BuChE is known to be generated from the common ancestor of vertebrate AChE, sharing of the similar properties between insect AChE2s and vertebrate BuChEs

may indicate an incidence of functional convergence regardless of their completely different origin and evolutionary time frame. Although the physiological function of ClAChE2 remains to be elucidated, ClAChE2 may not yet be specialized to gain distinct non-synaptic functions as judged by its CNS-specific distribution, membrane-anchored molecular property and the high catalytic efficiency that is still comparable to that of ClAChE1.

4.2 Functional specialization of ClAChEs and ClSChE

In some insects, one of the two AChEs (*A. mellifera*, *T. castaneum*) or one splice variant from a single *ace* locus (*D. melanogaster*) has been specialized to produce soluble forms or and gain non-neuronal functions (Kim, Cha et al. 2012; Lu, Park et al. 2012; Kim, Kwon et al. 2013). In other insects, including *B. germanica*, *C. pipiens*, *V. gracilis* (Weill, Fort et al. 2002; Huchard, Martinez et al. 2006; Kim, Choi et al. 2010; Kim, Kwon et al. 2013), however, both AChEs show relatively high catalytic activity and no apparent evidence for the presence of soluble AChE that is specialized for non-neuronal function has been reported. In addition to the distinct AChEs encoded from two different *ace* loci, various molecular forms of each AChE can contribute to the functional diversification of AChE. Considering this, the bed bug ChEs provide a unique example in that it has three different ChE loci encoding distinct ChEs (i.e., ClAChE1, ClAChE2 and ClSChE), with some of which having different molecular forms (i.e., membrane-

anchored vs. soluble ClAChE1), which are evolved to have putatively various functions. The membrane-anchored forms of both ClAChE1 and ClAChE2 appear to be specialized as synaptic AChEs whereas the soluble form of ClAChE1 and the ClSChE to acquire non-neuronal functions. Since the distribution of ClSChE is limited to the salivary gland, it was proposed to be involved in choline sequestration when injected into the tissue of animal host (Seong, Kim et al. 2012). In case of soluble ClAChE1, however, it still remains to determine its distribution and abundance in different tissues.

Presence of the soluble AChEs (or ChEs) putatively possessing non-neuronal functions in a range of bilaterians, including nematodes and insects, indicates that the acquisition of non-neuronal function of AChEs likely occurs before the evolution of insects. Within the class Insecta, however, the completely specialized soluble forms of AChE have been only found in the relatively evolved insects, such as the honey bee and fruit fly. In relatively more ancient insects, such as damselfly and cockroach, both AChE1 and AChE2 show relatively high catalytic AChE activity. In light of this, it is unusual that the bed bug has additional ChE (i.e., SChE), which appears to have completely non-synaptic function, along with catalytically active two AChEs. Taken together, it may be suggested that neuronal and non-neuronal functions are not yet likely specialized by one AChE between AChE1 and AChE2 at the early stage of insect evolution, although more studies on a variety of insects would be required to establish any evident trend.

CHAPTER 2

Molecular characterization of salivary gland specific-cholinesterase from the common bed bug, *Cimex lectularius*

Molecular characterization of salivary gland specific-cholinesterase from the common bed bug, *Cimex lectularius*

Abstract

The common bed bug, *Cimex lectularius*, has unique feature to have a cholinesterase expressed exclusively in the salivary gland (ClSChE). To infer the physiological function of ClSChE, I investigated the molecular form, tissue distribution patterns and enzyme properties of ClSChE. Under native conditions, ClSChE existed as the soluble dimeric form connected by a disulphide bridge or the soluble monomeric form. Immunohistochemistry of the salivary gland and duct confirmed that ClSChE was expressed in the gland cells. When phosphate-buffered saline (PBS) was artificially fed using the membrane feeding system, ClSChE was confirmed by western blot analysis using ClSChE-specific antibody to be secreted into PBS, further suggesting its secretive nature. To test the previous hypothesis that ClSChE is involved in choline sequestration when injected into the tissue of animal host, I conducted acetylcholine/choline assay using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The results showed that ClSChE has a weak catalytic activity to hydrolyze acetylcholine. Based on these findings, it was proposed that ClSChE is involved in the regulation of blood vessel compression of the host.

1 Introduction

In the nervous system of both vertebrates and invertebrates, acetylcholinesterase (AChE, EC 3.1.1.7) regulates the level of the neurotransmitter acetylcholine by hydrolyzing it into choline and acetic acid (Toutant 1989). Insect AChE is also a crucial enzyme in the insect nervous system, in which the cholinergic system is essential (Stojan, Marcel et al. 1998). In most insects, two acetylcholinesterases, AChE1 and AChE2, are encoded by two different loci, which was diverged from a duplication event that occurred long before the split of ecdysozoa (Gao, Kambhampati et al. 2002; Li and Han 2002; Baek, Kim et al. 2005; Kim, Jung et al. 2006; Lee, Kim et al. 2006; Kim, Cha et al. 2012).

Cimex lectularius, the common bed bug, is a hemtophagous pest, hosting most mammals, including human. Recently bed bugs have re-emerged in increasing number and caused serious problems particularly in the Europe, Australia, Canada and United states (Hwang, Svoboda et al. 2005).

The bed bug has unique feature of possessing three genes encoding different cholinesterase (ChE) types [AChE1, AChE2 and salivary gland-specific cholinesterase (SChE)] (Seong, Kim et al. 2012). According to phylogenetic analysis, all three bed bug ChEs were categorized into a large clade of ChEs. ClAChE1 was classified into the insect AChE1-type clade, whereas ClAChE2

was categorized into the insect AChE2-type clade (Seong, Kim et al. 2012). ClSChE was categorized into the clade containing the nematode and arachnid AChE1, which is closely located with an insect AChE1-type clade.

In preliminary data, ClAChE1 was widely distributed as a GPI-anchored dimeric form and a soluble dimeric or monomeric form in both the central nervous system (CNS) and peripheral nervous system (PNS) whereas ClAChE2 is found as a GPI anchored dimer in CNS. I also verified that ClAChE1 has higher catalytic activity than ClAChE2 through comparison of the catalytic properties of in vitro-expressed ClAChE1 and ClAChE2. However, ClSChE was predicted to be present as a soluble form mostly in the salivary gland and to have negligible catalytic activity (Seong, Kim et al. 2012).

Previous studies revealed that honey bee AChE1 (Kim, Cha et al. 2012) and pinewood nematode AChE3 (Kang, Lee et al. 2011) existed as soluble form and have little or low catalytic activity like ClSChE. Physiological property of honey bee AChE1 (AmAChE1) is highly similar to the pinewood nematode AChE3 (BxAChE3) (Kim, Cha et al. 2012). These soluble forms of ChEs were reported to be expressed in non-neuronal tissues and to have non-neuronal function (Kang, Lee et al. 2011). AChEs can produce soluble forms and have non-neuronal functions in some insects such as *A. melifera* and *T. castaneum* having one of two AChEs or in *D. melanogaster* having one splice variant from a single *ace* locus by alternative splicing (Kim, Cha et al. 2012; Lu, Park et al. 2012; Kim, Kwon et al.

2013). Although physiological role of non-neuronal AChE still remains unclear, a recent study suggested that a non-neuronal AChE from *T. castaneum* have some functions, such as female reproduction, embryo development and growth of offspring (Lu, Park et al. 2012). The soluble AChE of AmAChE1 and BxAChE3 play a role as a bio-scavenger such as vertebrate butyrylcholinestease (BChE) that provides a chemical defense (Kang, Lee et al. 2011; Kim, Cha et al. 2012).

Based on its negligible activity, ClSChE was previously suggested to be involved in the sequestration of choline or acetylcholine in the host blood when secreted into animal host during blood feeding (Seong, Kim et al. 2012). In this study, in an attempt to elucidate the physiological function of ClSChE in detail, I investigated the molecular properties and tissue distribution pattern of ClSChE. I also confirmed the salivary gland-specific expression profile and secretive nature of ClSChE. Finally, the hydrolytic/sequestration aspect of ClSChE was examined using the acetylcholine assay based on ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

2 Materials and methods

2.1 Insect and Chemicals

Bed bug population kept in insect breeding dishes was maintained at 27±1°C, 16-h light and 8-h dark photoperiod, and 70% relative humidity. The bed bugs were fed once a week on human blood using an in vitro rearing system as described previously (Yoon, Kwon et al. 2008).

Acetylthiocholine chloride, Choline chloride were purchased from Sigma Aldrich (St. Louis, USA) and Phosphate buffered saline (PBS) was bought from Caisson (North logan, UT, USA).

2.2 Sample preparation

2.2.1 Salivary gland protein

Salivary gland protein was extracted by homogenizing salivary gland isolated form bed bug using micro tissue grinder (Radnoti, Monrovia, CA, USA) in 0.1 M Tris-HCl (pH 7.8) with or without 0.5% Triton X-100 (v/v). The supernatant was collected from the homogenates after centrifugation at 12,000 g for 10 min at 4 °C. To remove excess lipids, the supernatant was filtered by glass wool and then stored at -80 °C until use.

2.2.2 Analysis of ClSChE secretion during feeding

Blood feeding by bed bugs was simulated by the membrane feeding system

using PBS. In brief, the modified smaller feeding cup (0.5 ml volume) was assembled and PBS was supplied instead of human blood. The PBS in the feeding cup was heated to 32±2 °C to stimulate feeding and 20 bed bugs were released over the membrane in the feeding cup. After allowed for sufficient feeding for 15 min, the remaining PBS was collected and concentrated by Ultra Amicon YM-30 (Millipore, Bedford, MA, USA) at 4 °C. The concentrated PBS was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the presence of ClSChE in the PBS was detected by western blotting as described in following section.

2.3 In vitro expression of ClSChE with a baculovirus expression system

In order to generate recombinant *Autographa californica* nucleopolyhedroviruses (AcMNPVs), the resulting transfer vectors were co-transfected with bAcGOZA DNA (Kim, Choi et al. 2010) into Sf9 cells maintained in TC-100 medium (WelGENE, Korea) supplemented with 10% of FBS at 27°C. Transfection was performed using Cellfectin (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions, and the recombinant viruses were purified by plaque assay of Sf9 cells as previously described (Oreilly, Brown et al. 1992). To exclude serum-derived esterase activity, Sf9 cells maintained in SF900-II serum-free medium (Invitrogen) were seeded onto 100-mm diameter tissue culture dishes at a density of 5×10^6 cells/dish,

followed by incubation at 27 °C for 30 min to allow cell attachment. Attached cells were washed twice with 3 ml of incomplete TC-100 medium and inoculated with 1 ml properly diluted viral stock. After incubation for 84 h at 27 °C, culture supernatants were collected by centrifugation at 5000 g for 15 min. Culture supernatants were concentrated using Ultra Amicon YM-30 (Millipore, Bedford, MA, USA) and then concentrated protein was concentrated with a YM-50 filter and was exchanged to buffer containing 0.1M Tris, 0.02M NaCl and 20% glycerol (pH 7.8). These samples were stored at - 80 °C until use.

2.4 Electrophoresis and Western blotting

To determine whether the ClAChEs molecular forms are soluble nature or membrane anchored nature, Salivary gland proteins (15μg) with or without triton X-100 were mixed with Native-PAGE sample buffer and separated by Native polyacrylamide gel electrophoresis (PAGE). Running condition of Native polyacrylamide gel electrophoresis (PAGE) (7.5%) is 120 V for 100 min in a cold chamber using a continuous Tris-glycine buffer system and the gel and running buffers contained 0.5% Triton X-100 (v/v). Protein samples were separated by this condition in triplicate. After Naïve PAGE, I conducted Western blotting as described below. To confirm that dimeric form of ClSChE was made by disulfide bridge and ClSChE secretion analysis during feeding, salivary gland protein sample (15μg) and samples for ClSChE secretion analysis were mixed with

sodium dodecyl sulphate (SDS)-PAGE sample buffer with or without 40 mM β-mercaptoethanol and separated by SDS-PAGE (4-12% gradient gel). One set of gels was conducted to coomassie staining whereas the other set was analysed by Western blotting as described below. Electrophoresis was conducted with a vertical electrophoresis unit (Novex mini cell, Invitrogen).

Gel for western blot analysis was electro-blotted to a Hybond-N nitrocellulose membrane (GE Healthcare, Pittsburgh, PA, USA) at 40V 200 mA for 1h 10 min. And the memebrane was conducted to step of blocking in phosphate-buffered saline (PBST) buffer containing 0.1% Tween-20 and 5% fat-free dry milk for 30min at room temperature. Each nitrocellulose membrane sheets was incubated for 3 h at room temperature or overnight at 4 °C with primary antibodies (1:5000-diluted ClAChE1 antibody and AChE2-specific antibody in PBST buffer) and washed three times with PBST buffer in 10-min intervals. The membranes were incubated with 1:5000-diluted horseradish-peroxidase conjugated anti-rabbit IgG secondary antibody (Pierce Bio-Technology, Rockford, IL, USA) for 1 h and washed three times with PBST buffer in 10-min intervals. And then the antigen-antibody complex on the bands was visualized using a chemiluminescence kit according to the manufacturer' instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.5 ClSChE Immunohistochemistry

To confirm the distribution of ClSChE in the salivary gland and salivary ducts,Whole-mount immunohistochemistry was conducted. After anesthetized by cooling, the Adult bed bugs were anatomized in PBS-0.2% Tween-20 (PBST). The salivary gland containing gland duct was fixed with Bouin's fixing solution for 20 min at room temperature. The tissue was washed in six times for 3 hours using PBST and dehydrated through a diluted ethanol series (10 min each in 20%, 40%, 60%, 80% and 100% ethanol diluted in PBST). And then it was stored in 100% aceton for 10min. It was rehydrated through the graded ethanol series (10 min each in 100%, 60% and 20% ethanol) and washed three times in PBST each for 10 min. The salivary gland was incubated in primary antibodies diluted 1:50 (ClSChE specific polyclonal antibodies) for 24 hours at 4 °C on a rotator. After three washes, the sample was incubated with secondary antibody (Anti-Rabbit Alexa568) diluted 1:400 in PBST at 4 °C for 24 hours. After three washes, the salivary gland was mixed with Vectasheild H-1200 (DAPI) (vector, Burlingame, CA, USA) for nucleus staining and mouned on slide glass. The whole-mount sample was photographed on Zeiss Laser Scanning Microscope (LSM) 710 (Carl Zeiss, Oberkochen, Germany). And then images were processed using Adobe Photoshop program (Adobe Inc, San Jose, CA, USA).

2.6 Acetylcholine assay

2.6.1 Standards of Acetylcholine/Choline

Choline chloride and acetylthiocholine chloride were dissolved in distilled water (D.W.). I prepared various concentrations of choline chloride (1, 5, 10, 20, 50 and 100 ppb) and acetylthiocholine chloride (0.2, 0.5, 1, 2, 5, 10, 20 and 50 ppb) by diluting in this solution. This solution was stored at room temperature.

2.6.2 Sample preparation

To determine whether the secreted ClSChE can sequester or hydrolyze acetylcholine/choline in the blood, bed bugs were stimulated to secrete ClSChE into the PBS containing physiological concentrations of choline/acetylthiocholine via the membrane feeding system. In brief, choline chloride and acetylthiocholine chloride were spiked into 0.5X PBS to the final concentration of 30 pmol/ml (30 fM) each, which is a 100-fold dilution from the known concentration (3.23 ± 0.23 nmol/ml) of acetylcholine in human blood (Hasegawa, Kunihara et al. 1982). Such dilution was needed to make the choline/acetylthiocholine concentration comparable to that of ClSChE used for the reaction (i.e., approximately 3-fold higher than the ClSChE concentration) so that sequestration or hydrolysis by ClSChE, if any, could be detected more clearly. Fifty bed bugs were placed into the membrane feeding system containing the choline/acetylcholine substrate solution, thereby allowing the incubation of secreted ClSChE with

choline/acetylcholine. As a positive control, a 1.5-μl aliquot of expressed ClSChE (approximately 0.9 ng, 10 fM final) was mixed with 1.5 ml of the substrate solution and incubated at 32±2 ℃ for 30 min using the membrane feeding system. As a negative control, the same amount of the expressed ClSChE was denatured by heating at 94 °C for 5 min and used for the incubation with the substrate solution. For the untreated control, only the substrate-PBS solution was incubated under the same conditions.

Following incubation at 32±2 ℃ for 30 min, all the substrate-PBS solutions were retrieved from respective feeding chamber and filtrated by Ultra Amicon YM-30 (Millipore) at 4 °C to remove any macromolecules, including the secreted ClSChE. Then, the filtrate was used for UPLC-MS/MS analysis.

2.6.3 UPLC-MS/MS conditions

UPLC–MS/MS analyses were carried out using an Acquity Ultra Performance LC system coupled to a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separation was achieved using a 1.7-μm particle size, 100mm×2.1mm Kintex HILIC column at a flow rate of 0.5 mL/min. The column temperature was set to 30 ◦C. Mobile phase A was 15mM ammonium formate in D.W., while mobile phase B consisted of 100% acetonitrile. Isocratic program was that the

composition of mobile phase was A and B (20:80) by linear gradient. The sample injection volume was 2 µl.

The chromatography system was coupled to the triple quadrupole mass spectrometer (MicroMass Quattro Premier XE) using positive electrospray ionization (ESI). The source conditions were set as follows: 250 °C source temperature, 0.40 kV capillary voltage, nebulizing gas flow rate of 3L/min, 400 °C heat block temperature. Also, drying gas was set at 15L/min. Dwell time was 0.1 sec, pause time was 0.003sec.

3 Results

3.1 Determination of ClSChE molecular forms

To determine whether the ClSChE molecular forms are soluble or membrane-anchored, I prepared protein samples from salivary gland using the extraction buffer with or without Triton X-100 (Fig.1). ClSChE protein was strongly detected at the positions of around 130-kDa (dimer) and 60-kDa (monomer) in both protein samples extracted in the presence and absence of Triton X-100. These findings suggested that ClSChE exists as soluble form in the salivary gland. The molecular weights (MWs) of ClSChE were analyzed by SDS-PAGE with or without β-mercaptoethanol reduction (Fig.1). A dimer of approximately 130-kDa was strongly detected under non-reducing conditions. After reduction with β-mercaptoethanol, a band of ClSChE (63.9 kDa) was visualized as a monomer whereas the dimeric form of ClSChE disappeared. According to this result, the dimeric form of ClSChE was determined to be connected with the disulfide bond.

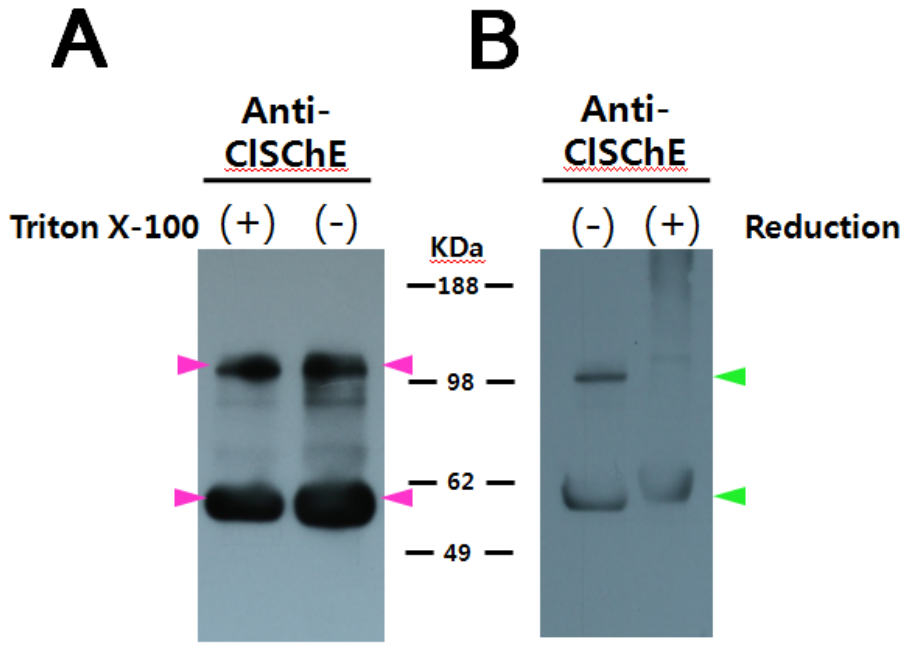
A
Anti-
ClSChE
Triton X-100 (+) (-)
B
Anti-
ClSChE
(-) (+) Reduction
KDa
188
98
62
49
C
S—S

Figure 1. Molecular characterization of ClSChE by SDS-PAGE and Western blot analysis. To investigate the soluble nature of ClSChE, protein sample was extracted from the salivary gland of bed bug with 0.1 M Tris-HCl buffer in the presence or absence of 0.5% Triton X-100 (A). To determine the multimer formation of ClSChE, protein was separated under reduction or non-reduction condition using β-mercaptoethanol (B). Schematic diagrams of the molecular structures of ClSChE. Soluble dimer and monomer of ClSChE are predominately expressed in salivary gland (C).

3.2 Localization of ClSChE within Salivary gland

To determine the distribution of ClSChE in the salivary gland of common bed bug, the salivary gland was isolated and fixed with Bouin's fixing solution for the whole mount immunohistochemistry. Immunohistochemistry using ClSChE-specific antibody revealed the typical localization patterns of ClSChE in the cells of salivary gland and salivary ducts. In these cells, ClSChE appeared as aggregates dispersed inside the cytosol (Fig. 2).

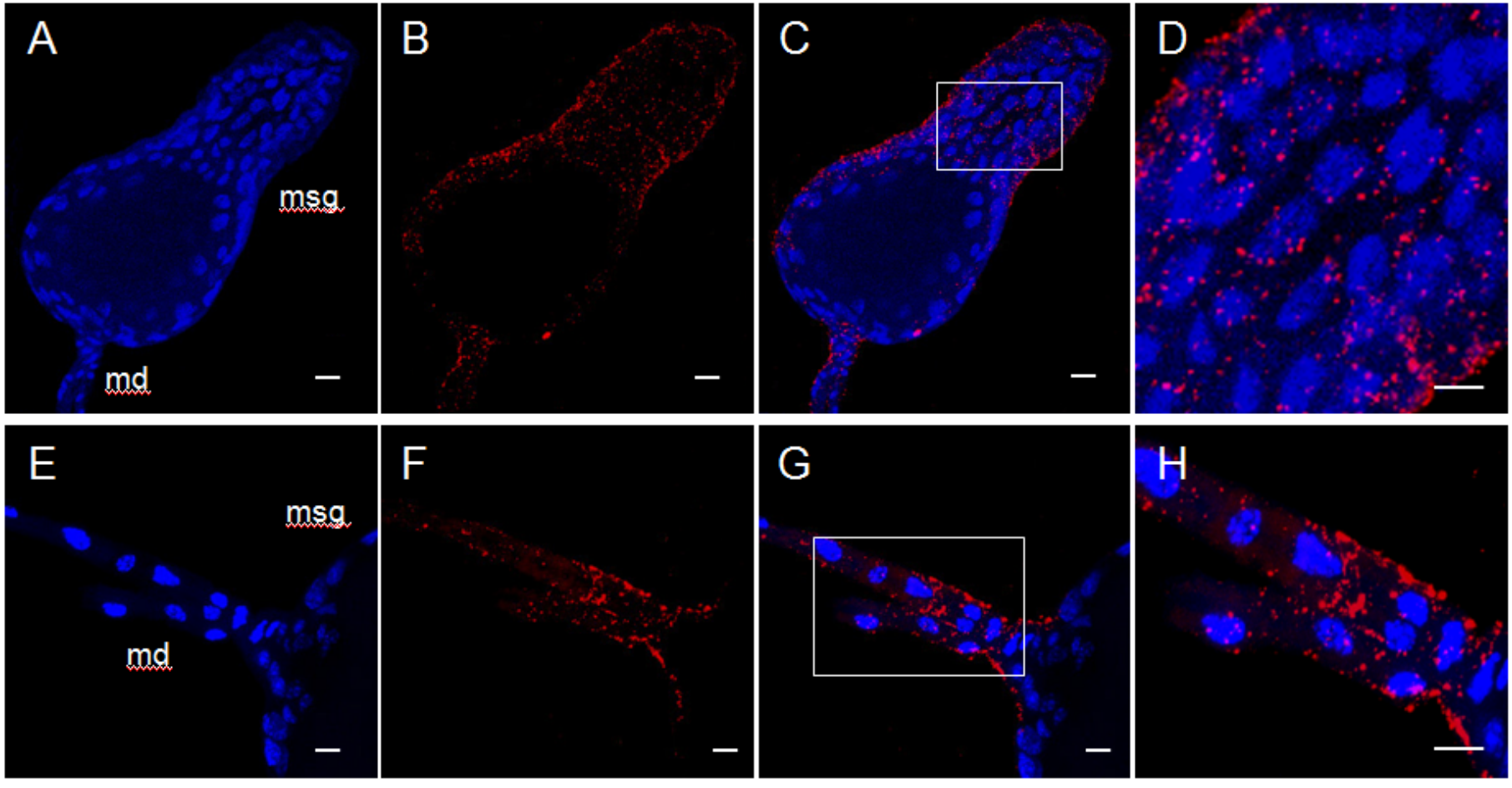
A
msg
md
B
C
D
E
msg
md
F
G
H

Figure 2. The whole mount of adult *Cimex lectularius*, showing the localization pattern of ClSChE in the main salivary gland (msg; A, B, C and D) and salivary gland duct (md; E, F, G and H). The nucleus were stained with DAPI in Vectasheild H-1200 (A and E). ClSChE (Red signal) was observed in the cell bodies which were placed in the whole salivary gland and salivary gland duct. The signals of nucleus staining and ClSChE were merged (C and G). The enlarged views of the white boxes were shown in D and H. The signal of ClSChE (Red) was observed in the around nucleus. The scale bar represents 10 ㎛.

3.3 Confirmation of ClSChE secretion during feeding

To confirm the hypothesis that bed bug secretes ClSChE during blood sucking, bed bugs were allowed to suck blood via the membrane feeding system. Following feeding, the blood was retrieved from the feeding chamber, centrifuged to remove the red blood cells and the resulting serum was separated by SDS-PAGE. Then, the presence of ClSChE in the serum was detected by western blotting using ClSChE-specific antibody (Fig. 3). In the western blot, the serum proteins exhibited strong non-specific interaction with the ClSChE-specific antibody, rendering the result inexplicable. To solve this problem, therefore, the blood was replaced with PBS in the feeding experiment. Following feeding PBS, the PBS was retrieved from the feeding chamber, concentrated and separated by SDS-PAGE with β-mercaptoethanol reduction. Then, the presence of ClSChE was detected by western blotting using ClSChE specific antibody (Fig. 3). Except for the unfed PBS control, ClSChE band was strongly detected at the position of around 60-kDa (monomer) in both the salivary gland extracts (positive control) and the PBS recovered from the feeding chamber. These findings revealed that soluble ClSChE expressed in the salivary gland was secreted into PBS, further suggesting that ClSChE is likely secreted into the host tissues when bed bugs suck blood.

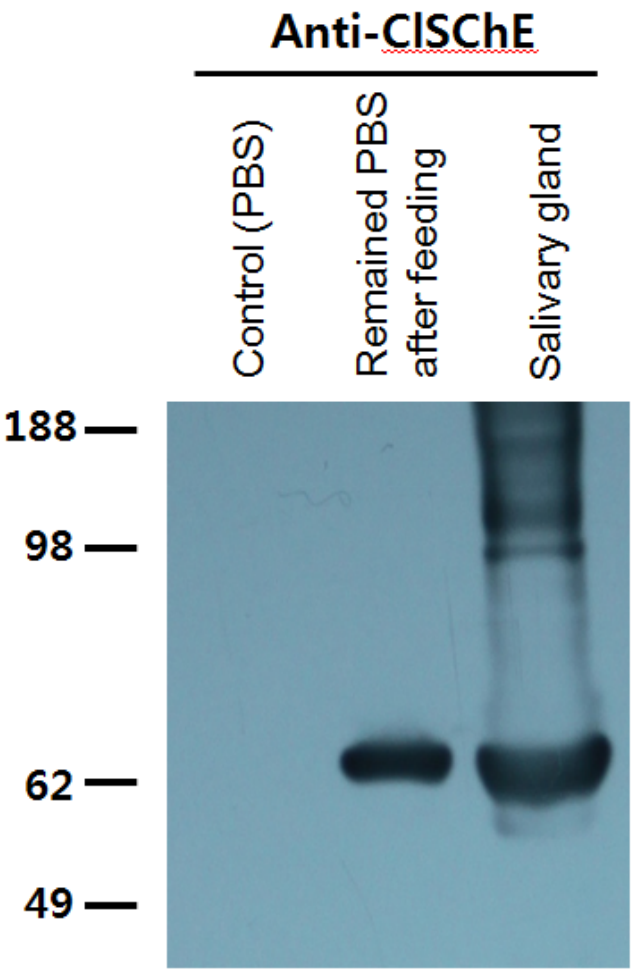

Figure 3. Confirmation of ClSChE secretion during sucking blood by SDS-PAGE and Western blot analysis. Samples are Phosphate buffered saline (PBS) as control, Remained PBS after feeding by bed bugs and salivary gland protein from bed bug with 0.1 M Tris-HCl buffer.

3.4 Acetylcholine assay of ClSChE

The choline peak (m/z 104.20 → 60.10) was detected at 5.12 min whereas the acetylcholine peak (m/z 146.10 → 87.05) was detected at 3.62 min. To estimate the actual amount of choline/acetylcholine from the peak area, calibration curve in a linear range was constructed the choline and acetylcholine concentrations below 100 and 50 ppb, respectively (Fig. 4). In the case of control, the nominal concentrations for choline and acetylcholine were 4.5 and 5.6 ppb, respectively, the actual concentrations determined from the calibration curve were 5.5 and 6.5 ppb, respectively. Therefore, all the test results were normalized with the actual control values under the assumption that no autohydrolysis of acetylcholine occurred.

To deduce the function of ClSChE, results obtained from of acetylcholine assay (positive control, negative control, treating bed bugs) were compared with control (Fig. 5). Compared with control, the negative control with inactivated ClSChE showed a similar result, in which the choline and acetylcholine concentrations were 5.2 and 6.4 ppb, respectively, indicating no hydrolysis or sequestration occurred. In contrast, the experiment using the ‘in vivo ClSChE’ revealed that most of acetylcholine was converted to choline after incubation but the combined amount of choline/acetylcholine before incubation was not significantly different from the amount retrieved from the filtrate (i.e., free

unbound choline/acetylcholine) after incubation. Likewise, the results of positive control using in vitro expressed ClSChE also showed similar results in that acetylcholine concentration was significantly reduced whereas choline concentration was greatly increased but without much difference in the total amount of choline/acetylcholine between before and after incubation. These results clearly indicate that ClSChE has hydrolytic function rather than sequestration function although the hydrolytic efficiency is not as high as common AChE.

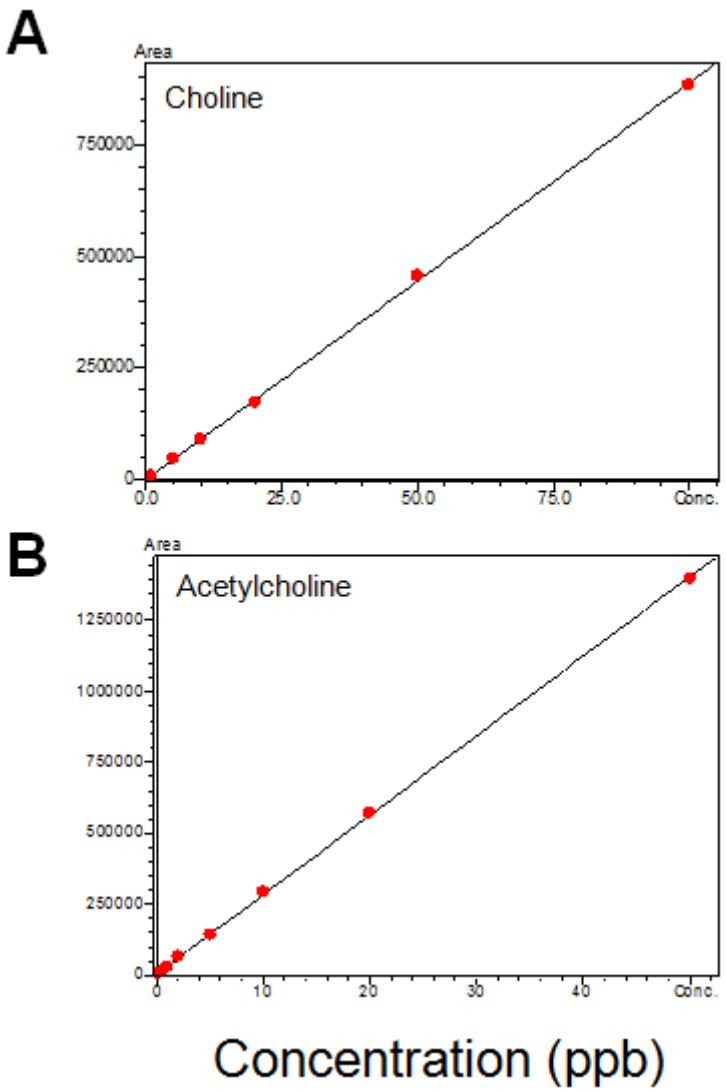

Figure 4. (A) Calibration curve for choline standards. (B) Calibration curve for acetylcholine standards.

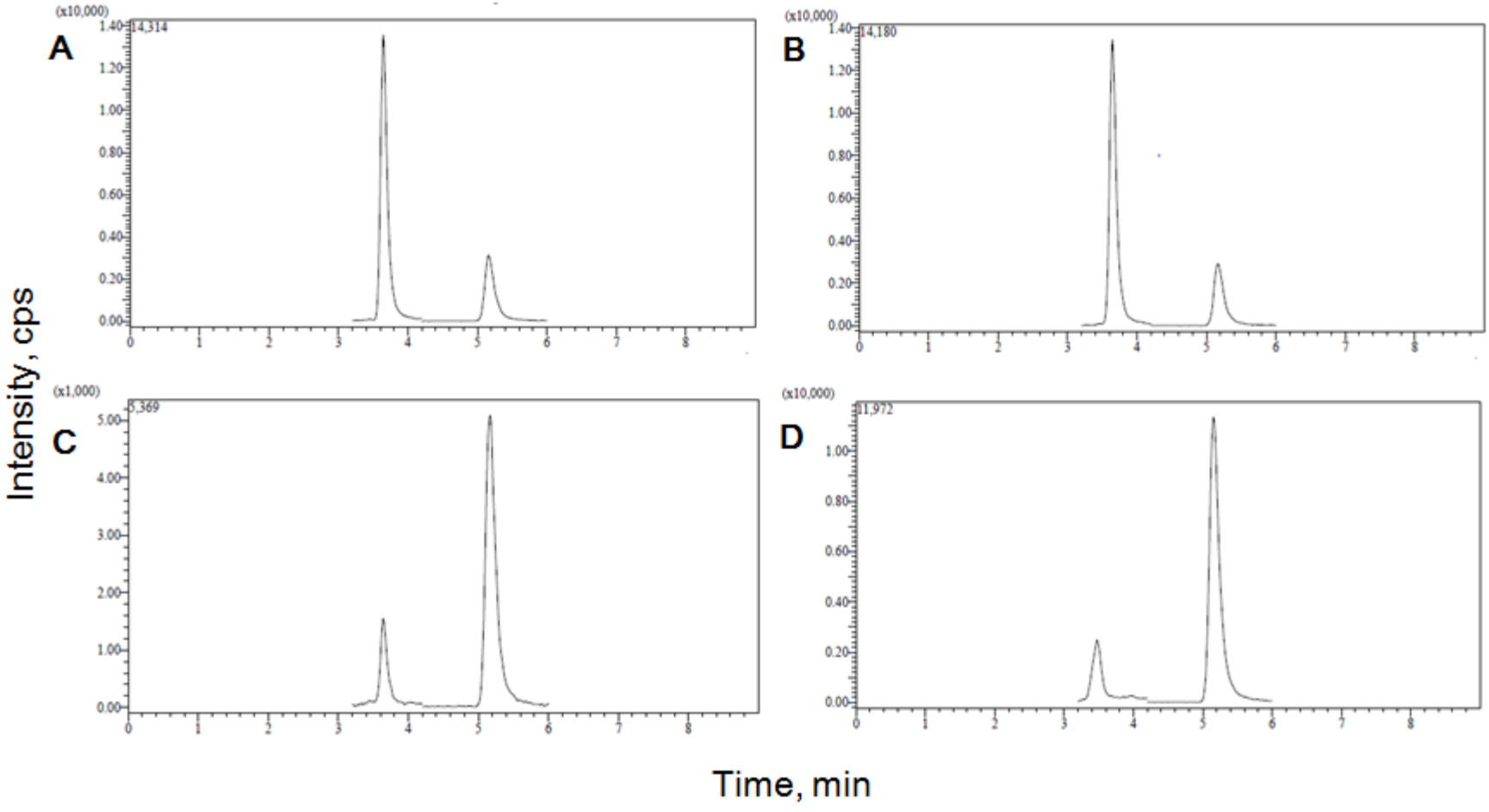
A
(x10,000)
14,314
B
(x10,000)
14,180
C
(x1,000)
5,369
D
(x10,000)
11,972
Intensity, cps
Time, min

Figure 5. MS chromatograms of acetylcholine and choline. Front peak is acetylcholine, rearward peak is choline. (A) control, (B) negative control including inactivated ClSChE, (C) positive control including expressed ClSChE and (D) experimental sample which was sucked by bed bugs.

Table 1. Converted amounts (ng) of choline and acetylcholine are measured by LC-MS/MS.

		Initial amount	Change	Feeding	Remaining	Total
in vitro (0.9 ng)	Acetylcholine	9.69 ng / 1.5ml	- 9.11 ng	-	0.59 ng / 1.5 ml	- 3.44 ng
	Choline	6.75 ng / 1.5ml	+ 5.66 ng	-	12.41 ng / 1.5ml	
in vitro (50 BBs)	Acetylcholine	9.69g / 1.5ml	- 4.36ng	- 5.17 ng / 700 µl	0.81 ng / 800 µl	0
	Choline	6.75 ng / 1.5ml	+ 14.50 ng	- 3.15 ng / 700 µl	18.10 ng / 800 µl	
Negative control	Acetylcholine	9.69 ng / 1.5ml	- 0.2 ng	-	9.59 ng / 1.5 ml	- 0.64 ng
	Choline	6.75 ng / 1.5ml	- 0.62 ng	-	6.13ng / 1.5 ml	

4 Discussion

4.1 Soluble and secretive nature of ClSChE

Molecular properties and tissue distribution patterns of ClSChE were examined in this study. As predicted from the fact that ClSChE does not have the GPI-anchoring sequence (Seong, Kim et al. 2012), ClSChE was determined to be soluble. ClSChE was present as two types of molecular forms: the soluble monomer and the soluble dimer that is connected by the disulfide bond. Such soluble nature is also found in other cases, including the honey bee (i.e., AmAChE1) and the pinewood nematode (BxAChE3) (Kang, Lee et al. 2011; Kim, Cha et al. 2012), whose functions were proposed to be non-neuronal.

Feeding simulation via the membrane feeding system using PBS uncovered that ClSChE is secreted into the external space, most likely into the host tissue, via the proboscis. Previous study on the bed bug sialome also found that the ClSChE accounted for 3.9% of bed bug saliva (Francischetti, Calvo et al. 2010). Taken together, ClSChE appears to be injected into the host tissue with other salivary components while blood sucking.

The imunohistochemistry result revealed that ClSChE was present around the nucleus in the cells of the salivary gland and gland duct (Fig. 2). Since ClSChE is soluble and secretive, the translated ClSChE in the salivary gland cells is likely sorted via the endoplasmic reticulum pathway, packaged into the vesicles and secreted into the lumen of the salivary gland. Considering this, the observed

aggregates of ClSChE in the immunohistochemistry appears to be the ClSChE transported by vesicles. The ClSChE that is concentrated inside the lumen of salivary gland is likely transported through the salivary duct and the salivary canal in the proboscis (Krenn and Aspock 2012). In summary, ClSChE expressed in the salivary gland and gland duct is injected into the host tissue through the salivary canal in the proboscis while sucking blood.

4.2 Putative physiological function of ClSChE

ClSChE showed little activity to choline substrates when the activity was measured by Elman's method in previous study. Lack of catalytic activity was suggested to be mainly due to the substitution of the highly conserved Ala201 (amino acid numbering of *Torpedo* AChE, hereafter) with Ser in ClSChE, which is one of the three key residues (i.e., Gly118, Gly119 and Ala201) forming the oxyanion hole (Seong, Kim et al. 2012). This substitution in the oxyanion hole appears to disrupt the hydrogen bonding between the NH group of key residues (i.e, Ala201) and the carbonyl oxygen of choline substrates, thereby destabilizing the formation of tetrahedral transition state intermediate and resulting in the reduction of catalytic efficiency.

Such lack of catalytic efficacy of ClSChE is a hypothesis that it may function as the choline/acetylcholine sequester protein when injected to the host tissue. Since human blood or body fluid contains small amount of choline/acetylcholine

(22.55 ± 3.97 and 3.23 ± 0.23 nmol/ml)(Hasegawa, Kunihara et al. 1982), which is known to be involved in blood vessel dilation (Kimura, Low et al. 2007), regulation of choline/acetylcholine concentration in the host tissue by sequestration via ClSChE would affect the blood flow or pressure in the local cutaneous tissue. To test the hypothesis, choline/acetylcholine assay was conducted to determine whether ClSChE can sequester the physiological concentrations of choline/acetylcholine. Unlike the expectation, total amount choline/acetylcholine associated with the filtrate was not changed following incubation with ClSChE, suggesting that these substrate molecules were not absorbed to ClSChE. Instead, acetylcholine was completely converted to choline after incubation with ClSChE, indicating its hydrolytic function. Therefore, though not measurable by the common colorimetric method (i.e., Elman's method), ClSChE was determined to retain a low level of acetylcholine hydrolytic activity. However, the kinetic properties of ClSChE remain to be determined.

Although the catalytic activity toward acetylcholine is much lower compared to typical AChE, it can hydrolyze the small amount of acetylcholine in the blood or body fluid when injected to the host tissue. Since acetylcholine can function as a blood vessel dilator, degradation of acetylcholine likely results in the contraction of blood vessels in the cutaneous tissue, which in turn increases the local blood pressure of the vessel, thereby facilitating blood sucking by bed bug.

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KOREAN ABSTRACT

빈대의 세가지 콜린에스터라제의 분자형태 및 효소학적 특성 연구

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초록

곤충 신경조직에서의 주요한 효소인 아세틸콜린에스터라제(AChE, EC 3.1.1.7)는 유기인계 및 카바메이트계 약제의 작용점이다. 대부분의 곤충의 경우 두 종류의 아세틸콜린에스터라제(AChE1, AChE2)가 서로 다른 유전자로부터 발현되는 것으로 알려져 왔다. 인간에게 불쾌감과 성가심을 유발하는 빈대는 흡혈곤충으로 최근 유럽, 오스트레일리아, 캐나다 그리고 미국 등 세계적으로 급증하고 있다. 이러한 빈대는 서로 다른 유전자로부터 발현되고 AChE1, AChE2, 침샘 특이적인 콜렌에스터라제(SChE)라 불리는 세 종류의 콜린에스터라제(ChE)가 존재한다는 독특한 특징이 있다. 본 연구에서는 빈대(*Cimex*

lectularius)의 세가지 콜린에스터라제(ChEs; ClAChE1, ClAChE2 and ClSChE)의 분자 형태 및 효소학적 특성을 규명하였다. ClAChE1 과 ClAChE2 의 조직 별 발현 양상을 확인 하고자 Native PAGE 후에 활성 스테이닝 결과, ClAChE1이 촉매반응을 일으키는 주요 효소로서 모든 조직에서 발현되며 반면에 ClAChE2는 중추신경계에서만 존재함을 확인하였다. 이들 두 아세틸콜린에스터라제는 모두 이황화 공유결합에 의한 2량체로서 GPI-anchor에 의해 세포막에 연결되어있었다. 또한 베큘로바이러스를 이용하여 발현한 두 아세틸콜린에스터라제의 동역학(kinetic)적 분석결과, ClAChE1이 아세틸콜린에 대하여 ClAChE2 보다 높은 효율의 촉매적 기능을 가지며, 이는 시냅스 후부의 전달에서 주요한 역할을 한다. ClAChE2는 부틸콜린과 다양한 기질에 대하여 높은 효율을 가지며 iso-OMPA에 의해 기질이 저해되었다. 약제 저해 실험 결과, ClAChE1이 모든 저해제에 대체로 민감함을 확인하였고, *in vivo*와 *in vitro*에서의 약제에 대한 저해의 상관관계 결과 ClAChE1이 유기인계와 카바메이트계 약제의 표적 효소임을 확인하였다.

침샘에 특이적으로 존재하는 ClSChE는 매우 미약하게나마 아세틸콜린을 분해하나 기능에 대하여 밝혀진 바가 없다. ClSChE는 친수성으로 이황화 공유 결합에 의한 2량체나 단량체 형태로 존재하는 것으로 나타났다. 빈대의 침샘과 침샘관을 면역조직화학염색에 의한 조직분포 양상결과, ClSChE는 세포에서 친수성 형태로 발현되며 침샘 내강을 통해 이동한다. 빈대가 흡혈 시에는 ClSChE가 빈대 구기 내부의 침샘관을 통해 외부로 분비됨을 확인하였다. ClSChE 역할을

확인하고자 아세틸콜렌/콜린 검정 결과, ClSChE는 약하지만 아세틸콜린을 가수분해 하는 촉매 활성을 가진 것으로 나타났다. 실험 결과들을 근거로 봤을 때, ClSChE는 흡혈과 관련되어 아세틸콜린에스터라제로서 비신경적 기능을 가질 것으로 사료된다. 이와 같은 결과는 곤충의 아세틸콜린에스터라제와 그의 진화에 대한 연구에 매우 유용한 정보라 사료된다.

검색어: 빈대, 분자 형태, 아세틸콜린에스터라제, 베큘로바이러스 발현, 동역학, 유기인계, 카바메이트계, 침샘 특이적인 콜린에스터라제, 면역조직화학염색법

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