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Characterization of Acetylcholinesterase from Korean Electric Ray and Comparison with *Torpedo Californica*

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This study has been undertaken to examine the acetylcholinesterase (AChE) of electric organ from korean electric ray(*Narke japonica*). Korean electric ray was caughted at Chungmu sea and transported to the laboratory, where electric organs were removed and stored at -70°C until used. Acetylcholinesterase(AChE) of electric organ was purified by affinity column that was prepared with dicaproyl-methylpyridinium linked to Sepharose 4B. Upon purification, the specific activities in Ellman unit were increased by 52 and 39 times for high salt soluble AChE (HSSE, 870.86 ΔOD/min/gram of tissue) and detergent soluble AChE(DSE, 105.42 ΔOD/min/gram of tissue), respectively. Each subunit of AChE separated by SDS polyacrylamide gel electrophoresis(SDS-PAGE) was transferred to immobilon P by western blotting and detected by mAbs raised against each subunit of AChE from electric organ of *Torpedo californica*. Collagenic tails of AChE from *Narke japonica* were identified by monoclonal antibody specific to collagenic tail of AChE from *Torpedo californica*, likewise 103Kd protein of AChE from *Narke japonica* was detected by monoclonal antibody specific to 103Kd of AChE from *Torpedo californica*. However, molar ratio of three subunits of AChE from *Narke japonica* is different from that of *Torpedo californica*. Furthermore, catalytic subunit of AChE from *Narke japonica* was not identified by monoclnal antibody specific to catalytic subunit of AChE from *Torpedo californica*. These results showed differences in molecular structure of AChE from *Narke japonica* and AChE from *Torpedo californica* eventhough they showed same enzymatic activities.

Key words : Acetylcholinesterase, *Narke japonica, Torpedo californica,* Monoclonal antibody, Purification

INTRODUCTION

Acetylcholinesterase (acetylcholine acetylhydrolase, AChE) controls neurotransmission through termination of signal released from nerve ending by rapid hydrolysis of neurotransmitter acetylcholine (ACh) at cholinergic synapse in central and peripheral nervous systems.

AChEs exist in multiple molecular forms (Bon *et al*, 1976; Inestrosa *et al.*, 1987; Massouli and Bon, 1987; Vigny *et al*, 1978) based on their subunit association (Assymetric:A, Globular:G) and hydrodynamic properties (High-salt soluble: HSSE, Low-Salt soluble: LSSE, Detergent-soluble: DSE). Asymmetric HSSE are associated with the basal lamina of the synapse (Brandan *et al.*, 1985; McMahan *et al*, 1978) and composed of catalytic subunits linked by disulfide bond to structural subunits such as collagenic tail with or without 103Kd protein(Massoulié, .1980). The Globular DSE, which are localized on the surfaces of the membrane in CNS or erythrocytes, exist as oligomers of a catalytic subunits (Massouli and Bon, 1982). Thus, molecular structural diferences in AChE seems to be related to cellular localization of this enzyme.

Despite of extensive studies of polymorphism of AChE, little is known about the physiological functions of different molecular forms of AChE. Because of their high density of synapse in the electric organ which is composed of modified muscle, the electric organs of the electric fish, *Torpedo* and *Electrophorus* have been used as rich sources of AChE and AChE receptors (Chang *et al.*, 1982 and Massouli and Bon, 1982). To investigate the molecular structure of AChE in the electric organ of electric fish, we used korean eletric lay (*Narke japonica*). We purified and characterized the AChE in the electric organ of korean electric ray (*Narke japonica*) and compared each subunit to that of *Torpedo californica* using monoclonal antibodies raised against each subunit of AChE from *Torpedo californica*.

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MATERIALS AND METHODS

Materials

Acetylthiocholin iodide, anti-mouse Ig G (whole molecule) alkaline phosphatase, conjugate, DTNB (5, 5'-dithiobis-2-nitrobenzoic acid), elastase, fast blue BB salt (Diazotized 4'-amino-2'-5'-diethoxy-benzanilide zinc chloride salt), iodoacetamide, lubrol WX, *streptomyces griceus* protease, piperazine-N,N'-bis (2-ethane-sulfonic acid),-naphthyl phosphate were from Sigma company (St. Louis, MO. USA)

Preparation of tissue

Korean electric ray *Narke japonica* was kindly provided from Lee dogil in Choongmu and *Torpedo californica* was purchased from Marinus company in USA. Elecric organs of *Narke japonica* and *Torpedo californica* were isolated and stored in liquid nitrogen until used.

Antibody preparation

Hybridoma cells donated by Dr. Chang at Columbia University in USA were grown in the Dulbecco's modified Eagle's Medium supplemented with 20% fetal bovine serum at 37°C in an atmosphere of 5% carbon dioxide-95% air. After the cells were confluent, the supernatants were collected and added equal volume of saturated ammonium sulfate solution and centrifuged at 20,000 g for 20 min at 4°C. The pellet was dissolved in one fourth of initial supernatant volume of water and dialysed against 10mM sodium phosphate buffer, pH 7.0, centrifuged again and the supernatants were aliquoted and stored at -70°C before used.

Extraction of high salt soluble and detergent soluble AChE

Electric organs from *Narke japonica* or *Torpedo californica* were homogenized in 200 ml of 10 mM PIPES, 10 mM EDTA, 0.1 mM NEM, 0.1 mM bacitracin, 10% antiprotease coctail and centrifuged at 25,000 g for 50 minutes at 4°C. 10 mM of iodoacetamide was added prior to homogenization. The residue (R1) of centrifugation was extracted two times by homogenization in 50 ml of 10 mM PIPES, 1.2 M NaCl, 1 mM EDTA, pH 7.0 and centrifugation at 140,000 g for 40 min. The supernatants (S2, S3) from above extraction contain the extract of high salt soluble AChE. The residue (R3) from S3 was washed two times with 100 ml of 10 mM PIPES buffer containing 10% antiprotease, pH 7.0, and extracted with 40 ml of 10 mM PIPES, 1.1% of Lubrol X by shaking for 3 hours at 4°C and centrifugation at 25,000 g for 1 hour at 4°C. The supernatant contains the crude extract of detergent soluble AChE and ACh receptor.

Purification of AChE by affinity column chromatography

The high salt and detergent extracted enzyme solution was purified by affinity column chromatography using 3 μM/ml of dicaproyl-methylpyridinium as a affinity ligand (Rosenberry *et al.*, 1972). The high salt soluble AChE, the supernatants (S2, S3), after dilution with an equal volume of 10 mM PIPES, pH 7.0, was loaded onto the column and washed with 50 ml of 0. 5 M NaCl in PE buffer (10 mM PIPES, 1 mM EDTA, pH 7.0), 1.0 M NaCl, PE buffer, 0.7 M NaCl, PE buffer. The elution of the retained enzyme was conducted by 100 μM decamethonium bromide, PE buffer with 0.7 M NaCl.

The detergent soluble AChE, the crude extract with 1% lubrol WX (S6), after dilution with an equal volume of 10 mM PIPES, 1 mM EDTA, 1 mM EGTA, pH 7.0, was loaded onto the column. The column was washed three stepwise: 30 ml of 10 mM PIPES, 1 mM EDTA, 0.3% lubrol WX, pH 7.0, 50 ml of 10 mM PIPES, 1 mM EDTA, 0.1% lubrol WX, pH 7.0, 30 ml of 10 mM PIPES, 1 mM EDTA, 0.05% Lubrol, pH 7.0 (PEL buffer). After the elution of ACh receptor with 150 M flexedil, the column was washed with 30 ml of 100 mM NaCl, PEL buffer, 30 ml of 400 mM NaCl, PEL buffer, 20 ml of 300 mM NaCl, PEL buffer. The detergent soluble AChE was eluted with 50 M decamethonium bromide. All procedures were conducted at 4°C.

Determination of AChE activity and AChE concentration

The activity of AChE was determined by the spectrophotometric method of Ellman (Ellman *et al.*, 1961) using 75 mM acetylthiocholine and 10mM of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in 0.1 M sodium phosphate buffer, pH 7.0 at room temperature. One Ellman unit is defined as the amount of enzyme which produces an increase in absorbance of 1 at 412 nm with a path length of 1 cm at 25°C. Protein concentrations of purified AChE were determined from the absorbance at 280nm with the use of E280 nm (0. 1%)=1.8 according to Rosenberry *et al.* (1972).

SDS-Polyacrylamide gel electrophoresis(SDS-PAGE) and western blot analysis

10 μg of purified enzyme was reduced by 35 mM DTT and each subunit was separated by SDS-PAGE according to Laemmli *et al.* (1970). Acrylamide concentration was 3.5% for stacking gel and 7.0-8.0% for separating gel. Reduced enzyme in 63 mM Tris. HCl, pH 6.6, 2% SDS, 7% SDS, 1 mM EDTA, 0.001%

bromphenol blue was loaded and the gel was run at 30 mA in 25 mM Tris, 192 mM glycine, pH 8.3, 0.1% SDS. Protein bands were visualized by staining with 0.04% Coomassie blue R, 25% methanol, 10% acetic acid. After electrophoresis, gels were transferred to immobilon P by western blotting (Towbin *et al.*, 1979). Each strip of membrane was incubated with monoclonal antibodies against each subunit of AChE from *Torpedo Califonica*, washed 4 times with 0.2% milk, 0.1% tween20 in 25 mM Tris, 0.1 M NaCl buffer, pH 7.4, for 5 minutes, incubated with anti-mouse Ig G (whole molecule) alkaline phosphatase conjugate for 1 hour at room temperature and the color was developed using α-naphthyl phosphate and fast blue BB salt as substrates.

RESULTS AND DISCUSSION

Extraction of AChE from the electric organ of korean electric ray

The AChE activities and the composition of AChE form from the electric organ of korean electric ray were shown in Table I. Among the extracted fractions, high salt soluble extract (HSSE; S2, S3) activity was about 69.66% and detergent soluble extract (DSE; S6) activity was 8.82% of total AChE activity. About 16.25% of total activity was shown in low salt soluble extract (LSSE; S1). This data was corresponds to earlier investigation of AChE from electric organ of *Narke japonica* (Sakai *et al.*, 1985).

Each HSSE and DSE fraction was purified by af-

Table I. Extraction of AChE from electric organ of *Narke japonica*

Supernatants	Enzyme form	Esterase activity (units/g)*	% of total protein
S1	Soluble, Globular	267.36	16.25
S2+S3	HSS, Asymmetric	1145.88	69.66
S4 + S5	HSS Asymmetric	86.58	5.26
S6	DS Globular	145.14	8.82

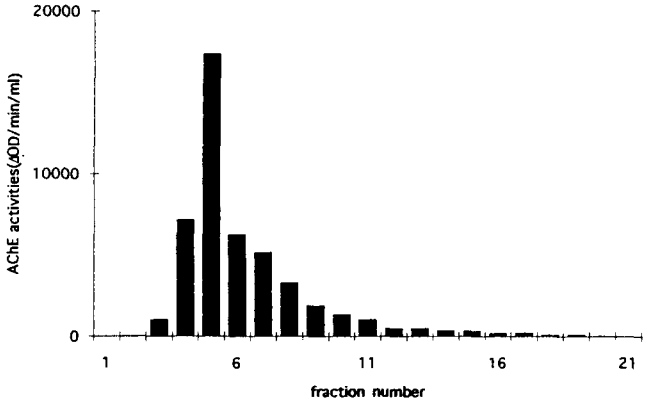

Fig. 1. The purification profile of high salt soluble AChE (HSSE) of electric organ of korean electric ray. HSSE was extracted with 1.0 M NaCl, 1 mM EDTA, 10 mM PIPES, pH 7.0 buffer. Extracted AChE was purified by affinity chromatography of Sepharose 4B-dicarproylmethylpyrinidium and eluted by 100 M decamethonium bromide. The activities of each purified fractions were assayed by Ellman method as in Methods.

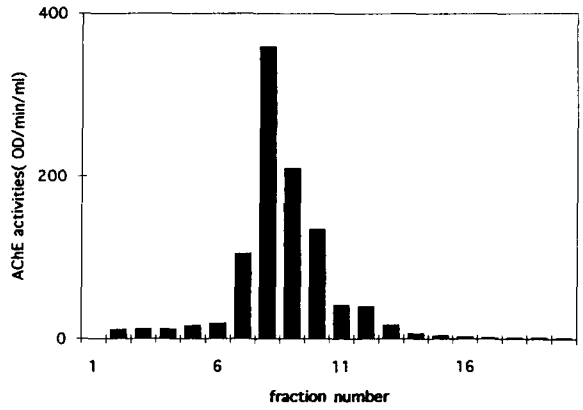

Fig. 2. The purification profile of detergent soluble AChE (DSE) of electric organ of korean electric ray. DSE was extracted with 1.1% Lubrol, 10 mM PIPES, pH 7.0. Extracted AChE was purified by affinity chromatography of Sepharose 4B-dicarproylmethylpyrinidium and eluted by 100 μM decamethonium bromide. The activities of each purified fractions were assayed by Ellman method.

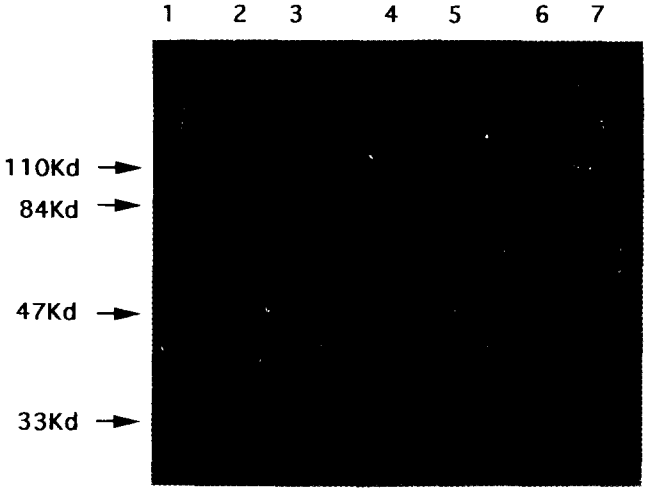

Fig. 3. SDS-PAGE of each subunit of purified AChE from electric organ. Purified samples were pooled and vacuum dialyzed and incubated with 35 mM dithiothreitol in 2% SDS. Electrophoresis was on a 7.5% polyacrylamide gel containing 0.1% SDS and run in 25 mM Tris, 192 mM glycine, 0.1% SDS. lane 1) size markers, lane 2) HSSE of Narke japonica (NJ), lane 3) HSSE of Torpedo californica (TC),lane 4) DSE of NJ, lane 5) DSE of TC, lane 6) ACh receptor of NJ, lane 7) ACh receptor of TC.

finity column chromatography using 3 μM dicaproylmethylpyrinidium-Sepharose 4B as a ligand. 79.19 % of AChE of HSSE fraction was bound to affinity gel, and 96% of gel-bound AChE (870.86ΔOD/min/gram tissue, Fig. 1) was eluted. In the case of DSE fraction, 92.11% of AChE was bound to affinity gel, and 80.4% of gel-bound AChE (105.42ΔOD/min/gram tissue, Fig. 2) was eluted.

Table II. Relative molecular weight and molar ratio of subunits of AChE purified from electric organ of *Narke japonica and Torpedo californica*

	Electric tissue	HSSE catalytic	HSSE collagenic	HSSE second-structural	DSE catalytic
Subunit size	*Narke japonica*	64Kd	58Kd	103Kd	60Kd
	Torpedo californica	67Kd	50Kd	103Kd	62Kd
Molar* ratio	*Narke japonica*	3	165	25	
	Torpedo californica	9	90	16	

*Each band on the SDS-PAGE was scanned with densitometer and divided by their molecular weight.

Subunit composition

In order to examine the subunit composition of AChE from the electric organ of korean electric ray (*Narke japonica*), dithiothreitol treated AChE was analyzed by SDS-PAGE, and each subunit of AChE from the electric organ of *Narke japonica* was compared with that of *Torpedo californica*. As shown in Fig. 3, high salt soluble extracts (HSSE) from both *Narke japonica* and *Torpedo californica* contains collagenic tails, 103Kd protein, catalytic subunit while detergent soluble extracts (DSE) from both *Narke japonica* and *Torpedo californica* contains catalytic subunit only. However, this two different electric organ showed the differences in the molecular weight of catalytic subunits and collagenic tails of HSSE as shown in the lane 2 and the lane 3 in Fig. 3. And the molar ratio of each subunit of HSSE from *Narke japonica* appeared to be different from that of HSSE from *Torpedo californica* (Table II). Molar ratio of AChE subunit from *Narke japonica* was quite comparable to that of electric eel (Massoulié and Bon, 1982).

Subunit analysis

Each subunit of AChE from *Narke japonica* was analyzed by western blot hybridization using mono-

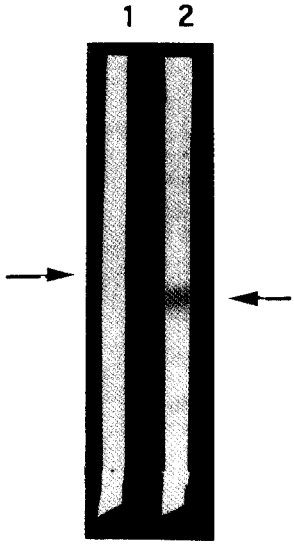

Fig. 4. Western blot analysis of collagenic tail subunits of high salt soluble AChE. Purified samples were separated by SDS-PAGE and transferred to immobilon P by Western blotting. Membranes were cut into narrow strips and incubated with monoclonal antibodies (mAb) as first antibodies, and anti-mouse Ig G (whole molecule) alkaline phosphatase conjugate as a second antibody. Color detection was carried out by α-naphthyl phosphate and fast blue BB salt in pH 9.5. Collagenic tail subunit detected with mAb (mAEH-TA) raised against collagenic tail of HSSE from Torpedo californica. Arrow indicates the collagenic tail. lane 1) Narke japonica, lane 2) Torpedo californica.

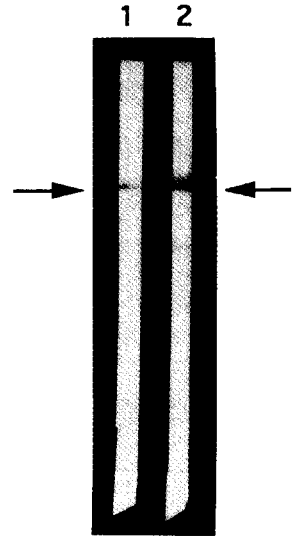

Fig. 5. Western blot analysis of 103Kd protein of high salt soluble AChE. Purified samples were separated by SDS-PAGE and transferred to immobilon P by Western blotting. Membranes were cut into narrow strips and incubated with monoclonal antibodies (mAb) as first antibodies, and anti-mouse Ig G (whole molecule) alkaline phosphatase conjugate as a second antibody. Color detection was carried out by α-naphthyl phosphate and fast blue BB salt in pH 9.5. 103Kd structural subunit detected with mAb (mAEH-103) raised against 103Kd subunit of AChE fromTorpedo californica. Arrow indicates the 103Kd protein. lane 1) Narke japonica, lane 2) Torpedo californica.

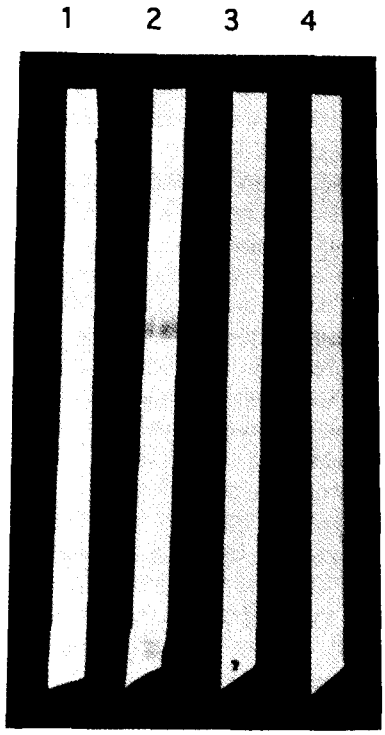

Fig. 6. Western blot analysis of catalytic subunits of high salt soluble enzyme (HSSE) and detergent soluble enzyme (DSE) purified from *Narke japonica* (NJ) and *Torpedo californica* (TC). Purified samples were separated by SDS-PAGE and transferred to immobilon P by Western blotting. Membranes were cut into narrow strips and incubated with monoclonal antibody raised against catalytic subunit of HSSE of TC as a first antibody, and anti-mouse Ig G (whole molecule) alkaline phosphatase conjugate as a second antibody. Color detection was carried out by α-naphthyl phosphate and fast blue BB salt as substrates in pH 9.5. Monoclonal antibodies (mAEH-CA) raised against active site of catilytic subunit of HSSE from TC were used. lane 1) HSSE of NJ, lane 2) HSSE of TC, lane 3) DSE of NJ, lane 4) DSE of TC.

clonal antibodies rasised against each subunit of AChE from *Torpedo californica*.

Collagenic tails of AChE from *Narke japonica* were identified by monoclonal antibody to collagenic subunit of AChE from *Torpedo californica* (mAEH-TA), but the molecular size of collagenic tails of *Narke japonica* appeared to be different from that of *Torpedo californica* (Fig. 4). Furthermore, mAEH-TA has much less affinity to the same amount of the collagenic tail of *Narke japonica* than that of *Torpedo californica*. Considering the fact that collagenic tails are known as dominant structural subunit of HSSE of AChE(Chang *et al.*, 1982), molecular structure of AChE from Narke japonica might not be identical to that of *Torpedo californica*.

103Kd protein, which is another structural subunit of AChE from *Narke japonica* was identified by monoclonal antibody to 103Kd protein of AChE from *Torpedo californica* (mAEH-103) as shown in the Fig. 5. Both AChE of Narke japonica and AChE of *Torpedo californica* showed 103Kd protein, but *Torpedo californica* contained more 103Kd protein than *Narke japonica*.

In the case of catalytic subunits, catalytic subunits of HSSE and DSE from *Torpedo californica* were identified monoclonal antibody to active site center of HSSE from *Torpedo californica* (mAEH-CA), whereas catalytic subunits of HSSE and DSE from *Narke japonica* was not identified (Fig. 6). On the contrary, catalytic subunits of HSSE and DSE from *Narke japonica* was identified by monoclonal antibody to peripheral site of catalytic subunit of *Torpedo californica* (mAED-CP, mAEH-CP) (Data not shown).

These data indicated that peripheral site of catalytic subunits of both HSSE and DSE from *Narke japonica* share epitope with that of *Torpedo californica*, however, active site center of catalytic subunit of AChE from *Narke japonica* contained different epitope from that of *Torpedo californica*.

CONCLUSION

We purified the high salt soluble AChE and detergent soluble AChE from Korean electric ray. Separated subunits of AChE show common epitope-recognizing regions with the subunit of AChE from *Torpedo californica*. However, there are also strong probabilities of structural differencies in the active sites of the catalytic subunits of AChE between two *Torpedo* AChEs.

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