

# Molecular Characterization of Maize Acetylcholinesterase. A Novel Enzyme Family in the Plant Kingdom<sup>1</sup>

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Acetylcholinesterase (AChE) has been increasingly recognized in plants by indirect evidence of its activity. Here, we report purification and cloning of AChE from maize (*Zea mays*), thus providing to our knowledge the first direct evidence of the AChE molecule in plants. AChE was identified as a mixture of disulfide- and noncovalently linked 88-kD homodimers consisting of 42- to 44-kD polypeptides. The AChE hydrolyzed acetylthiocholine and propionylthiocholine, but not *S*-butyrylthiocholine, and the AChE-specific inhibitor neostigmine bromide competitively inhibited its activity, implying that maize AChE functions in a similar manner as the animal enzyme. However, kinetic analyses indicated that maize AChE showed a lower affinity to substrates and inhibitors than animal AChE. The full-length cDNA of maize *AChE* gene is 1,471 nucleotides, which encode a protein having 394 residues, including a signal peptide. The deduced amino acid sequence exhibited no apparent similarity with that of the animal enzyme, although the catalytic triad was the same as in the animal AChE. In silico screening indicated that maize *AChE* homologs are widely distributed in plants but not in animals. These findings lead us to propose that the AChE family, as found here, comprises a novel family of the enzymes that is specifically distributed in the plant kingdom.

A well-known biological function of acetylcholine (ACh) is as a neurotransmitter in animals and insects. ACh serves to propagate an electrical stimulus across the synaptic junction. At the presynaptic neuron end, an electrical impulse triggers release of ACh, which accumulates in vesicles into the synaptic cleft via exocytosis. ACh then binds to an ACh receptor (AChR) on the postsynaptic neuron surface, and the ACh-AChR binding induces subsequent impulses to the postsynaptic neuron. Finally, ACh, which is released again by the receptor into the synaptic cleft, is rapidly degraded by acetylcholinesterase (AChE; E.C.3.1.1.7; Kelly et al., 1979; Dunant et al., 1980; MacIntosh, 1981). Meanwhile, ACh has been recently identified in a number of non-neuronal tissues in animals, fungi, bacteria, and plants (Wessler et al., 2001; Horiuchi et al., 2003). Although plants lack a nervous system, both ACh and ACh-hydrolyzing activity have been widely recognized in the plant kingdom (Evans, 1972; Fluck and Jaffe, 1974a, 1974b; Hartmann and Kilbinger, 1974; Verbeek and Vendrig, 1977; Lees et al., 1978; Momonoki and Momonoki,

1991). The ACh-hydrolyzing activities in plant tissues can be measured by the appearance of radioactive acetate following [1-<sup>14</sup>C]ACh hydrolysis (Momonoki, 1992), by the appearance of thiol group following acetylthiocholine (ASCh) hydrolysis (Momonoki, 1997), and by histochemical detection of ASCh hydrolysis (Momonoki, 1997; Momonoki et al., 1998). Moreover, the ACh-hydrolyzing activity in plant tissue is inhibited by neostigmine bromide, which is known as a specific inhibitor of the animal AChE (Momonoki, 1992). Thus, it is widely accepted that an ACh-hydrolyzing enzyme, namely AChE, is widely distributed in the plant kingdom.

When a plant is moved from the vertical orientation to a horizontal position, the stem shows curvature as it returns to a vertical position by response acting against gravistimulus. According to investigations of asymmetrically growing maize (*Zea mays*) shoots, endogenous indole-3-acetic acid (IAA; Bandurski, 1980; Bandurski et al., 1986), D-Glc (Momonoki, 1988), and IAA-inositol (Momonoki, 1988) were all localized mainly in the lower half of the horizontally oriented shoots. The IAA accelerates the growth of the lower side of the shoot, and, as a result, the shoot curves to a vertical position. These findings implied the probable existence of a potential-gating system, which would allow the asymmetric hormone and substance distributions following the stimulus. On the other hand, maize seedlings that were treated first with neostigmine bromide did not respond to the gravistimulus (Momonoki, 1997). Furthermore, after the stimulus, the AChE activity was distributed asymmetrically at the interface between the stele and

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the cortex of the gravistimulated maize seedlings (Momonoki, 1992). An asymmetric solute distribution of the AChE activities in maize seedlings (Momonoki et al., 1998) and in rice (*Oryza sativa*) seedlings (Momonoki et al., 2000) was also inhibited by neostigmine bromide. The role of AChE in animal nervous systems has been clearly demonstrated by the effects of its inhibitors. Neostigmine bromide is an AChE inhibitor having no inhibitory effect on IAA metabolism in plants (Ballal et al., 1993). Therefore, these findings strongly suggest that AChE plays a role in the gravity response of plants, and a hypothetical ACh-mediated system was proposed as a candidate for the potential-gating regulator. This system might achieve cell-to-cell transport of the hormone and other substances between the plant cells and might propagate a membrane depolarization. Significant increases of AChE activity in plant tissues appeared not only after gravity stimuli but also in response to heat stress (Momonoki and Momonoki, 1993). Heat tolerant plants, such as yard-long bean (*Vigna sinensis* var. *sesquipedalis*), cucumber (*Cucumis sativus*), and radish (*Raphanus sativus*), which are native tropical zone plants, showed high AChE activity in their node, stem, pulvini, and root after heat stress (Momonoki and Momonoki, 1993).

At the animal neuromuscular junction, an electrical impulse causes an influx of  $\text{Ca}^{2+}$  from outside the membrane via voltage-gated channels, and then the  $\text{Ca}^{2+}$  acts as a trigger for ACh release. Based on fluorescence analysis,  $\text{Ca}^{2+}$  was localized in the cortex cells surrounding the vascular system, in the epidermis, and in adhering peripheral cortical cells. A simultaneous increase of AChE activity and  $\text{Ca}^{2+}$  in the endodermal cells between the stele and the cortex in the maize seedlings was observed following heat stress, indicating that the plant ACh-mediated system might respond to  $\text{Ca}^{2+}$  signals in a manner similar to the animal system (Momonoki et al., 1996). Recently, it was reported that the bamboo (*Phyllostachys bambusoides*) shoot (especially the upper portion), whose growth rate is very rapid, contains significant levels of ACh and ACh synthesis capacity (Horiuchi et al., 2003). Most reports concerning plant ACh and its related molecules have suggested that an ACh-mediated system might play a role in the response to environmental stimuli.

The existence and biological functions of AChE as a constituent of an ACh-mediated system in plant tissues has been hypothesized based on indirect evidence such as measurement of ACh-hydrolyzing activity and inhibitory tests using anti-AChE reagents. However, direct evidence of the molecule(s) responsible for ACh hydrolysis was missing, and the corresponding gene had not yet been identified. In this study, the AChE protein was purified from etiolated maize seedlings, and the cDNA encoding the maize AChE was cloned and sequenced. Moreover, the enzymatic properties of the purified maize AChE were characterized and compared with those of electric eel AChE as a representative of animal AChE. The

purified maize AChE exhibited enzymatic properties similar to those of the electric eel AChE, except in the relative affinities to substrates and inhibitors. The maize AChE possesses a consensus sequence from the lipase Gly-Asp-Ser-Leu (GDSL) family, which is distinct from the carboxylesterase family consensus sequence that characterizes the animal AChE. Homologous maize AChE genes are widely distributed in the plant kingdom, based on *in silico* screening results, but similar genes were not found in any animal, fungal, or bacterial databases. Therefore, the AChE family, as found in this study, comprises a novel AChE family that is specifically distributed in the plant kingdom.

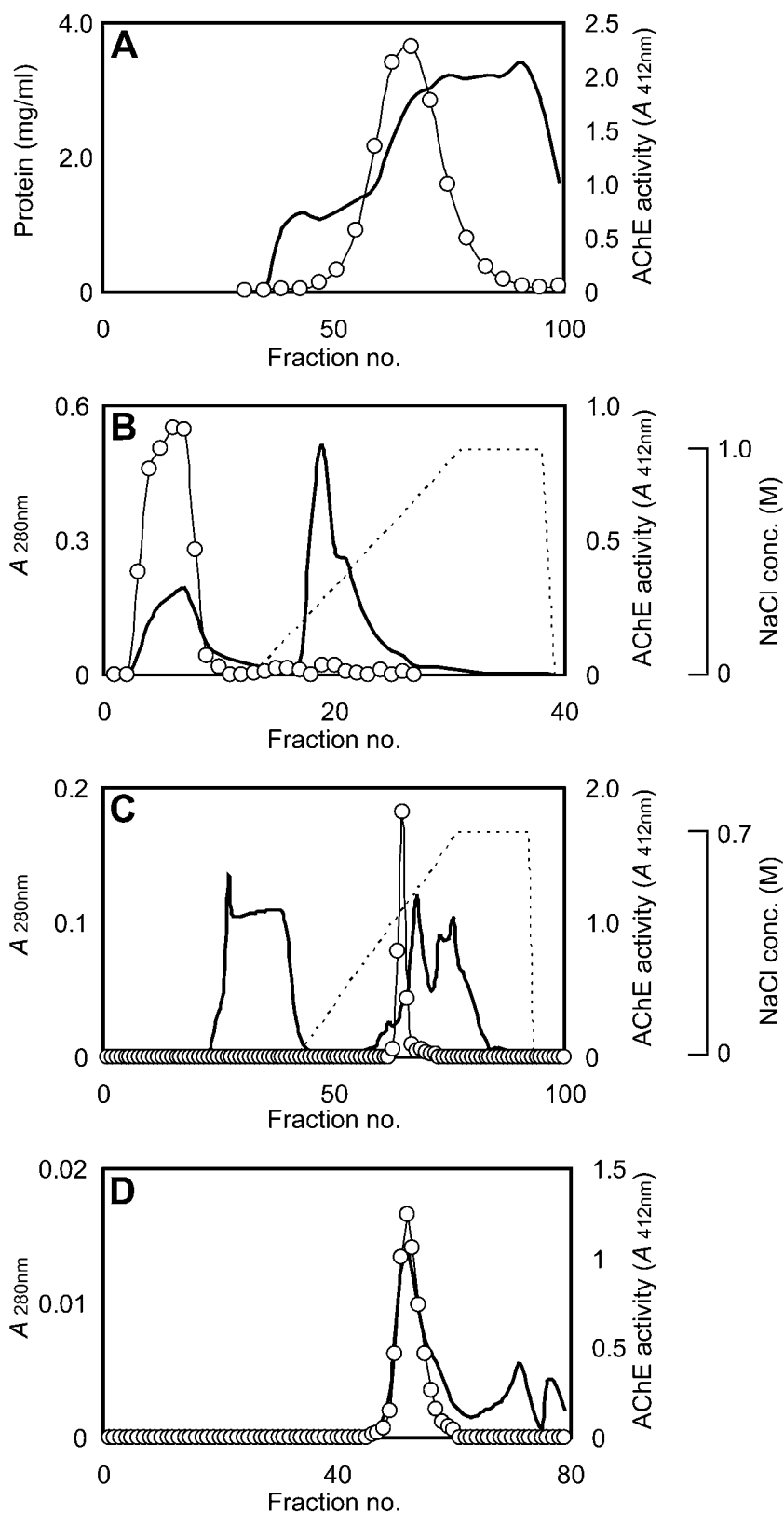
## RESULTS

### Purification of AChE from Maize Seedlings

Ten batches of the crude extract of the maize seedlings were combined and subjected to the following chromatographic purification procedure. The extract was applied to a Sephadex G-200 gel filtration column ( $2.5 \times 75$  cm; Amersham Biosciences, Uppsala; Fig. 1A). The active fractions were collected and applied to a Poros HQ/20 column ( $4.6 \times 100$  mm; PE-Applied Biosystems, Foster City, CA) equilibrated with 20 mM phosphate buffer, pH 7.0. The AChE activity was found in flow-through fraction, as shown in Figure 1B. The active fractions were collected and applied to a Poros HS/20 column ( $4.6 \times 100$  mm; PE-Applied Biosystems) equilibrated with 20 mM phosphate buffer, pH 5.0, and the adsorbed proteins were eluted with a linear NaCl gradient ranging from 0 to 0.7 M, as shown in Figure 1C. The active fractions were eluted as the major peak. The fractions were further purified with a Hilo Superdex 200 pg 16/60 column ( $1.6 \times 60$  cm; Amersham Biosciences) equilibrated with 20 mM phosphate buffer, pH 7.0, as shown in Figure 1D. The purification procedure parameters are summarized in Table I. We could not assay to determine the precise AChE activities for the crude enzyme and the ammonium sulfate fractions because these samples were colored, probably due to a pigment from the plant tissues, and this pigment would interfere with the colorimetric assay. After this series of purification procedures, the yield from 2 kg of maize seedlings was 0.8  $\mu\text{g}$  of protein having a total activity of 0.07 units.

### Molecular Composition of Maize AChE

The protein was then analyzed by PAGE under nondenaturing conditions (native PAGE), and the purified AChE preparation showed two predominant bands having different Coomassie Brilliant Blue staining intensities (designated as the U and L forms, Fig. 2A) and several minor bands. Both the U and L bands showed AChE activity, as indicated by staining using ASCh as a substrate (Fig. 2A), and production of the colored product was inhibited by adding an AChE-specific inhibitor, neostigmine bromide, indicating



**Figure 1.** Purification of the maize AChE by column chromatography. Line, Absorbance at 280 nm; line with circles, AChE activity. A, A typical elution profile of maize AChE on a Sephadex G-200 column equilibrated with phosphate-buffered saline. B, A typical elution profile of maize AChE on a Poros HQ/20 column equilibrated with 20 mM phosphate buffer, pH 7.0. C, A typical elution profile of maize AChE on a Poros HS/20 column equilibrated with 20 mM phosphate buffer, pH 5.0. D, A typical elution profile of maize AChE on a Hiload Superdex 200 pg 16/60 column equilibrated with 20 mM phosphate buffer, pH 7.0.

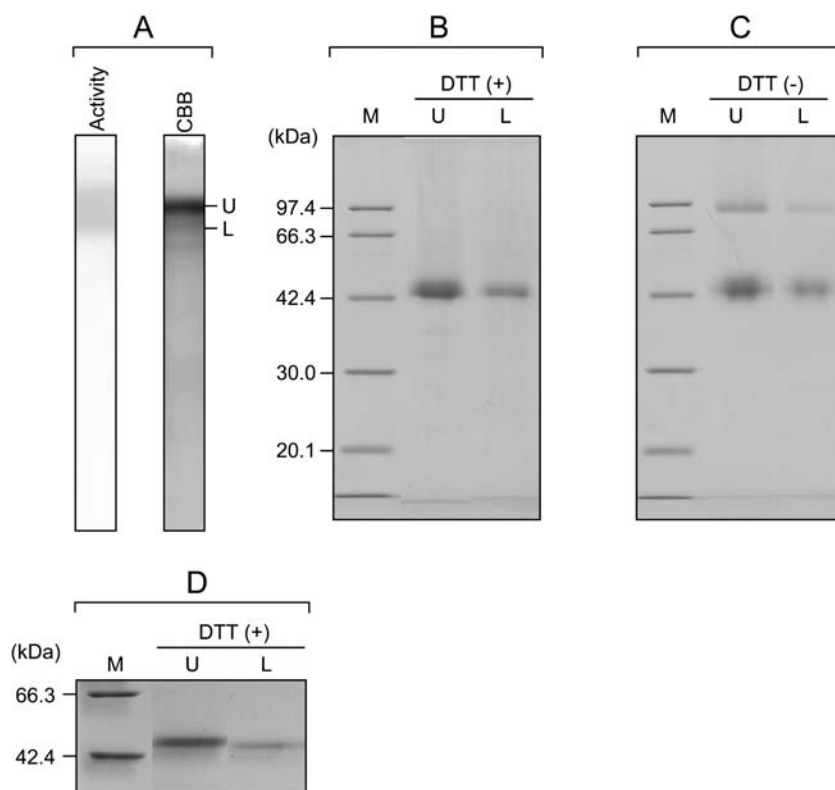
**Table 1.** Summary of the purification of maize AChE

Purification Steps	Activity	Total Protein	Specific Activity	Purification-fold	Yield
	<i>units</i>	<i>mg</i>	<i>units/mg</i>		<i>%</i>
Sephadex G-200	5.45	15.59000	0.4	1.0	100
Poros HQ	3.33	5.37000	0.6	1.8	61.09
Dialysis	3.12	3.71000	0.8	2.4	57.27
Poros HS	0.80	0.06530	12.3	35.0	14.67
Superdex 200	0.07	0.00079	82.5	236.0	1.20

that each band certainly contained AChE. The native PAGE gel slices corresponding to AChE protein bands, which appeared as U and L forms, were boiled with SDS-PAGE sample buffer in the presence of dithiothreitol (DTT) for 5 min and then subjected to SDS-PAGE using 12.5% polyacrylamide gels. As shown in Figure 2B, each extract migrated as a diffuse band corresponding to a molecular mass of between 42 and 44 kD on SDS-PAGE. On the other hand, each extract displayed two bands on SDS-PAGE in the absence of DTT, one sharp band with an apparent molecular mass of 88 kD and another diffuse band with a molecular mass of approximately 42 to 44 kD, as shown in Figure 2C. These results seemed to indicate that both the U and L forms of AChE exist as a mixture of 88-kD dimers consisting of disulfide-linked 42- to 44-kD polypeptides and a 42- to 44-kD monomer. Diffuse bands are often observed in the electrophoretic banding patterns of glycoproteins on SDS-PAGE, and

sharpness of the bands may vary with the polyacrylamide concentration of the gels. Indeed, both of the 42- to 44-kD polypeptides corresponding to the U and L forms seen on reduced SDS-PAGE were stained with the periodic acid Schiff reagent, indicating that both polypeptides are certainly glycosylated (data not shown). To compare the electrophoretic mobilities of the bands in more detail, the U and L forms of AChE extracted from the native PAGE gels were applied to reducing SDS-PAGE using 15% polyacrylamide gels. As shown in Figure 2D, each extract exhibited a single band on the gels, as with the 12.5% gels, but these bands were sharper. The L form of AChE migrated farther than did the U form on the 15% gel, indicating that the L form of AChE consists of a smaller subunit, compared with the U form of AChE. The N-terminal amino acid sequences for the 42- to 44-kD U- and L-form proteins were identical, NH<sub>2</sub>-AGAGGDCHFPAVFNFGDSNS. This result implied that the U and L

**Figure 2.** Native and SDS-PAGE analysis of the purified maize AChE. A, Native PAGE of the partially purified maize AChE. Separated proteins were stained with Coomassie Brilliant Blue (CBB) and AChE activity stain (Activity). B, SDS-PAGE (12.5%) of the extractions (U and L, which correspond to bands in A) from the native PAGE gels in the presence of DTT. The lane indicated by M is molecular mass standards. C, SDS-PAGE (12.5%) of the extractions from the native PAGE gels in the absence of DTT. D, SDS-PAGE (15%) of the extractions from the native PAGE gels in the presence of DTT.



forms of AChE are the same gene products with different posttranslational modifications, which may consist of the deletion of several residues from the C termini and/or different degrees of the glycosylation.

Thus far, we have presumed that the purified AChE existed as mixture of the monomeric and dimeric forms of the 42- to 44-kD glycosylated polypeptides. However, the maize AChE eluted as a single peak during all chromatographic purification procedures, and both the U and L forms of the AChE protein ran as a single band on the native PAGE. These behaviors showed an obvious discrepancy with the original postulation. The purified enzyme was subjected to analytical gel filtration on a Superdex 200 HR10/30 column equilibrated with phosphate buffer, pH 7.0, containing 0.15 M NaCl and 10% glycerol. As a result, the AChE activity eluted as a single peak with an elution volume of 14.2 mL, and its apparent molecular mass was estimated as 67 kD. The AChE preparation did not display any other active peaks eluting in ranges corresponding to either twice or half the size of the 67-kD protein on gel filtration, indicating that the maize AChE exists as a single molecular species without any differently sized forms. Therefore, it was concluded that the U and L forms of AChE were composed of a mixture of different dimeric forms: a disulfide-linked homodimer and a noncovalently linked homodimer, each of which consists of 42- to 44-kD polypeptides. Because the U-form protein was identified as the predominant form by Coomassie Brilliant Blue staining on the native PAGE, we speculated that the U-form protein is a mature enzyme, and the L form is a Golgi-mediated processing intermediate form or a partially digested form of the mature enzyme, resulting from proteolytic digestion in the plant tissues or during the purification procedures. The molecular mass of the enzyme was estimated by analytical gel filtration, and this estimate did not agree with the 88-kD band observed on SDS-PAGE in the absence of DTT. The apparent molecular mass (67 kD) of the enzyme on analytical gel filtration was probably a low estimate resulting from a specific interaction between the enzyme and the gel filtration resin and/or a compact conformation of the dimer forms.

#### Characterization of Purified Maize AChE

The cholinester-hydrolyzing enzymes are usually classified into AChE and butyrylcholinesterase (BChE), according to their substrate and inhibitor specificity. To examine the substrate specificity of the maize AChE among thiocholinesters containing acyl chains of different lengths, the enzymatic activity was tested using ASCh, propionylthiocholine (PpSCh), and S-butyrylthiocholine (BSCh) as substrates with the 5-5'-dithiobis-2-nitrobenzoic acid (DTNB) method. To compare the enzymatic characteristics of the maize enzyme with those of the animal enzyme, the commercially purchased electric eel AChE (Sigma-Aldrich, St. Louis) was used. The activity of maize and electric

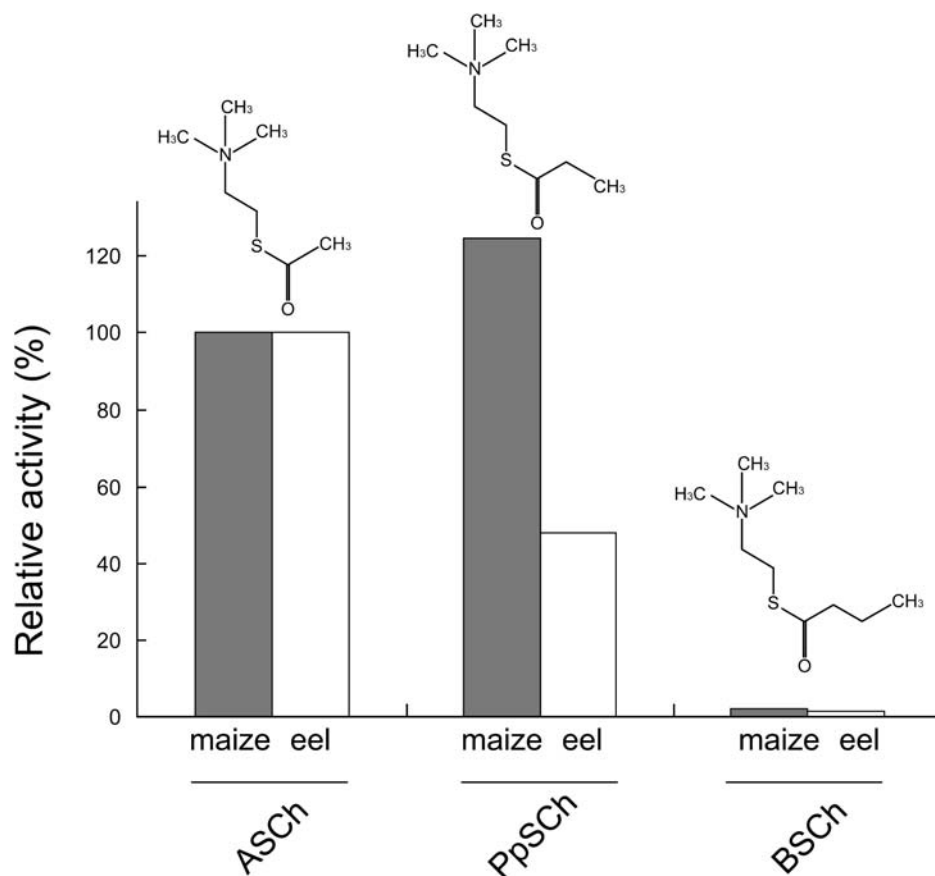
eel AChE against ASCh was taken to be 100%. As shown in Figure 3, the maize AChE exhibited 1.2-fold increased enzymatic activity against PpSCh compared with that against ASCh, while the electric eel AChE showed 0.5-fold activity against PpSCh than against ASCh. Both of the AChEs exhibited extremely low enzymatic activity against BSCh. Thus, the substrate selectivity of the maize AChE was estimated as PpSCh > ASCh  $\gg$  BSCh, and that of the electric eel AChE was ASCh > PpSCh  $\gg$  BSCh. Furthermore, the kinetics of the maize and eel AChEs were examined by using ASCh and PpSCh as substrates (Table II). The maize AChE showed a slightly higher  $K_m$  value of 4.7 mM for ASCh, compared with 3.1 mM for PpSCh. Moreover, these values were higher than the  $K_m$  values of 0.16 mM for ASCh and 0.84 mM for PpSCh, as determined for the electric eel AChE. Furthermore, the maize AChE displayed relatively low  $V_{max}$  values of 1.7  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  for ASCh and PpSCh, compared with the  $V_{max}$  values of 312.5  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  for ASCh and 263.2  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  for PpSCh on the electric eel AChE.

Neostigmine bromide is known as a specific inhibitor of the animal AChE, as it binds to the catalytic site of the enzyme (Standaert, 1990), and it also inhibits plant AChE (Momonoki, 1992; Ballal et al., 1993). To examine the inhibitory effects of neostigmine bromide on the hydrolyzing activities of maize AChE, the kinetics of the maize and electric eel enzymes against neostigmine bromide were examined by Dixon plots (Dixon, 1953), as shown in Figure 4. As a result, the neostigmine bromide inhibited the hydrolysis of the ASCh and PpSCh in a typically competitive manner, with apparent inhibition constant values ( $K_i$ ) of  $3.1 \times 10^{-6}$  M and  $4.7 \times 10^{-6}$  M, respectively, for maize AChE. The maize AChE exhibited relatively high  $K_i$  values, compared with  $K_i$  values for the electric eel AChE, which were  $9.0 \times 10^{-9}$  M for the ASCh hydrolysis and  $5.7 \times 10^{-9}$  M for PpSCh hydrolysis.

#### Nucleotide Sequence Coding for the Maize AChE cDNA

The 42- to 44-kD polypeptide of the U-form AChE, which was separated by SDS-PAGE in the presence of DTT, was digested with *Staphylococcus aureus* V8 protease and generated proteolytic fragments were applied for N-terminal amino acid sequence analyses. Of the several derivative fragments, two probable internal sequences were yielded, NH<sub>2</sub>-YFSQALYTF-DIGQNDITSSY and NH<sub>2</sub>-VEAIPDLME. A tBLASTn search against the maize database using these amino acid sequences identified a partial-length maize mRNA nucleotide sequence (accession no. AY107095) as a candidate. A partial open reading frame, which covers all short amino acid sequences determined for the purified AChE protein, was identified in the mRNA sequence. However, the sequence does not contain termination codon, although it possesses a presumed initiating ATG codon. Hence, we determined the complete nucleotide sequence corresponding to

**Figure 3.** Comparison of substrate selectivity of the maize and eel AChE. ASCh, PpSCh, and BSCh were used as substrates, and the activities were measured by the DTNB method as described in "Materials and Methods." The activity of maize and electric eel AChE against ASCh was taken to be 100%. The structural diagrams of the substrates are illustrated at the top of each column.



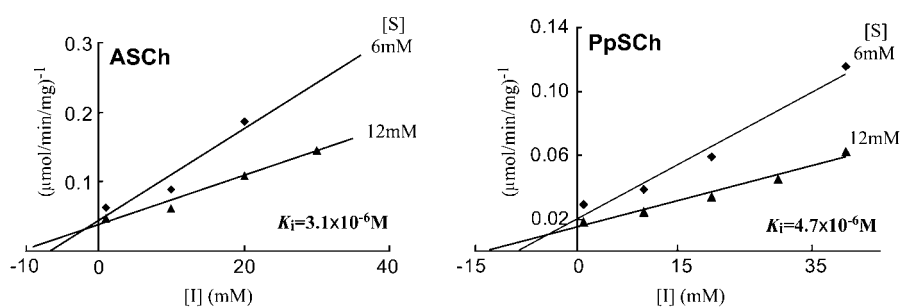
its full-length cDNA in this study. The cDNA library was prepared by 5'- and 3'-RACE using total RNA extracted from 5-d-old seedlings, prepared according to the same procedure used for the AChE protein purification. The primers used were Mmn-2 for the 5'-RACE (5'-ATCCAGAAGTACCTTCCTCC-3') and Sgn-1 for the 3'-RACE (5'-ACAGAAGCCAACCTCGTCTAC-3'), and these were designed to obtain fragments that overlapped with each other in a 235-bp region. The purified 5'-RACE (770 bp) and 3'-RACE (940 bp) PCR products were subcloned, and then each nucleotide sequence was determined using the primers designed from the partial-length mRNA sequence (Table III). The determined nucleotide sequence consists of 1,471 bp. The sequence contains a putative entire open reading frame, which consists of 1,182 bp and encodes 394 amino acid residues. The N-terminal amino acid sequence for the purified AChE corresponds to the sequence starting from

Ala-30 of the amino acid sequence deduced from the nucleotide sequence of the cDNA. The N-terminal amino acid sequences determined for the V8 protease-derived fragments were identical to those deduced from the DNA sequence for Tyr-171 to Tyr-190 and for Val-199 to Glu-208, indicating that this cDNA certainly encodes the maize AChE protein. The sorting server TargetP (Emanuelsson et al., 2000) predicts that the N terminus of the deduced sequence of the maize AChE precursor protein contains a signal sequence consisting of 29 residues, which routes the protein to secretory pathway with a probability of 93%. This prediction is supported by the observation that 29 amino acid residues are removed from the mature AChE. The calculated molecular mass of AChE without these 29 N-terminal amino acids is 39,722.3 D, with an pI of 7.18. The apparent molecular masses of the AChE subunits were estimated to be approximately 42 to 44 kD by SDS-PAGE in the presence of DTT,

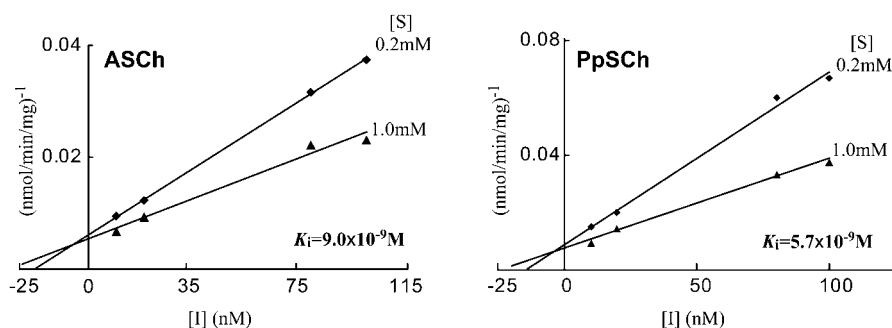
**Table II.** Kinetic data of the maize and electric eel AChE against substrate using ASCh and PpSCh

	ASCh		PpSCh	
	$K_m$	$V_{max}$	$K_m$	$V_{max}$
	<i>mM</i>	$\mu\text{mol min}^{-1} \text{mg}^{-1}$	<i>mM</i>	$\mu\text{mol min}^{-1} \text{mg}^{-1}$
Maize	4.7	1.7	3.1	1.7
Eel	0.2	312.5	0.8	263.2

## Maize



## Electric eel



**Figure 4.** Inhibitory efficiency of neostigmine bromide on ASCh and PpSCh by the maize and eel AChE. The  $K_i$  values were determined by Dixon plot, as described in "Materials and Methods."

which differed from the calculated value. In the deduced sequence, six consensus sequences indicating *N*-glycosylation sites, N-X(S/T), were identified, and this prediction was supported by the periodic acid Schiff staining results. Furthermore, 10 Ser, five Thr, and five Tyr residues were predicted to be probable phosphorylation sites by the NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>). Thus, the discrepancy observed between estimated and calculated molecular masses might be due to post-translational modifications such as glycosylation.

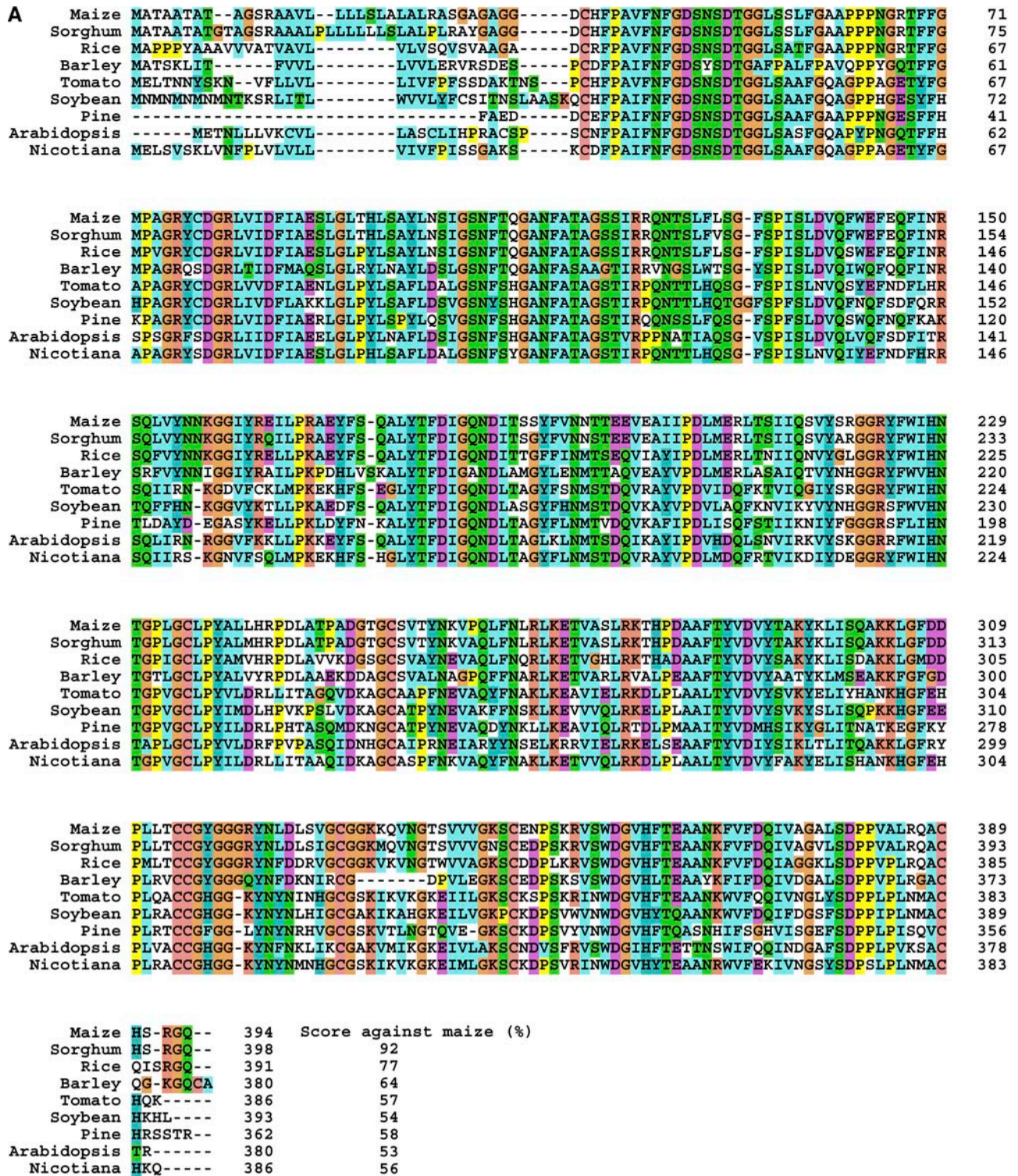
The protein domain architecture derived from the deduced amino acid sequence of the maize AChE was examined by computer-assisted protein family analysis using the protein family database Pfam (<http://www.sanger.ac.uk/Software/Pfam/>). The result showed that the maize AChE contains a conserved putative lipase GDSL family domain at residues 41 to 373. To search for genes homologous to maize AChE in other eukaryotes, the deduced amino acid sequence of the maize AChE was submitted to the tBLASTn search against The Institute for Genomic Research (TIGR) Unique Gene Indices (<http://tigrblast.tigr.org/tgi/>), which contain tentative consensus and singleton EST

sequences from 29 animals, 24 plants, 15 protists, and eight fungi. Significantly related sequences were found only in plant indices containing the sorghum (*Sorghum bicolor*), rice, barley (*Hordeum vulgare*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), soybean (*Glycine max*), pine (*Pinus ponderosa*), and Arabidopsis (*Arabidopsis thaliana*) plants. Comparison of the amino acid sequences deduced from these sequences using a ClustalX multiple sequence alignment analysis indicated that the amino acid sequences of probable AChE gene products in these plants are highly conserved, and they exhibit 53% to 92% identity against the amino acid sequence of maize AChE (Fig. 5). tBLASTn analysis against the bacterial genomic sequence database was also performed on the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov/BLAST/>), but no homologous genes were found in this database.

To analyze the exon and intron organization of the maize AChE in the genomic DNA, the 28 nucleotide sequences of the genomic DNA fragments partially encoding the maize AChE precursor were gathered by tBLASTn analysis using the deduced amino acid sequence from the maize genomic DNA library

**Table III.** Nucleotide sequences of primers used for this study

Forward	Sequence	Target	Reverse	Sequence	Target
Sgn-1	5'-ACAGAAGCCAACCTCGTCTAC-3'	531-550	Mmn-2	5'-ATCCAGAAGTACCTTCCTCC-3'	746-765
Sgn-3	5'-CAAAGGCGGTATCTACAAGG-3'	556-575	Mmn-4	5'-TAGATGTGAGCCTCTCCATC-3'	701-722
Sgn-5	5'-TTCTCCCAGGCATTTACACCTTCG-3'	599-623	Mmn-6	5'-CGGTCAAACGACATCACCTCAAGC-3'	1,306-1,330
Sgn-7	5'-GCAGGGAGAGGGAAATGG-3'	72-89	Mmn-8	5'-GTCAGCAGAGGGTCGTCG-3'	1,006-1,023
Sgn-9	5'-ACCCTCTGCTGACATGCTG-3'	1,011-1,029	Mmn-10	5'-GCACTGAGGTGAGTCAGTC-3'	360-378



**Figure 5.** ClustalX analysis of the amino acid sequences of the products of maize *AChE* gene homologs found in plant gene indices. The *AChE* homologs were identified by a tBLASTn analysis against TIGR Unique Gene Indices. A, Alignment of the amino acid sequences deduced from the *AChE* homologous genes. The amino acid sequence identities between the maize *AChE* and each gene product are indicated as percentages in the bottom column. B, Phylogenetic tree based on the ClustalW analysis. The tentative consensus sequence numbers used in this analysis are as follows: sorghum, SbGI TC95805; rice, OsGI TC237385; barley, HvGI TC120185; tomato, LeGI TC116906; soybean, GmGI TC195186; pine, PGI TC39002; Arabidopsis, AtGI TC209133; Nicotiana, NbGI TC2824.



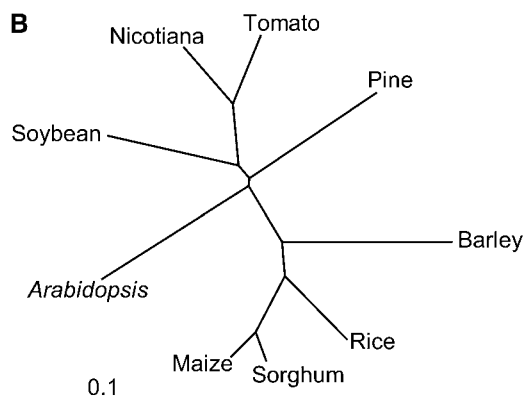


Figure 5. (Continued.)

database, and then the sequence fragments were assembled into single contiguous sequences by the CAP3 Sequence Assembly Program (Huang and Madan, 1999). By comparing the sequences of cDNA and genomic DNA, the AChE gene was found to consist of five exons and four introns on the maize genomic DNA, according to the GT-AG rule.

## DISCUSSION

The AChE protein was purified from the crude extract of 5-d-old etiolated maize seedlings. Consequently, 0.8  $\mu\text{g}$  of AChE protein having a specific activity of 82.5 units/mg was purified from 2 kg of seedlings. The purified AChE separated into two forms having different electrophoretic mobilities and different Coomassie Brilliant Blue and AChE-activity staining intensities on native PAGE. There were dominant U and minor L forms. The U and L forms of AChE ran as a broadly diffuse band with an apparent molecular mass of 42 to 44 kD on reducing SDS-PAGE, while on the nonreducing SDS-PAGE, it ran as two bands with apparent molecular masses of 42 to 44 and 88 kD, respectively. Because the purified AChE eluted as a single active peak with an apparent molecular mass of 67 kD upon analytical gel filtration, the native AChE protein apparently has only one molecular size, rather than several differently sized forms. Therefore, the U and L forms of AChE might exist as two types of dimeric forms: disulfide-linked and noncovalently linked dimers, each consisting of 42- to 44-kD subunits. Simultaneous existence of the disulfide-linked and noncovalently linked homodimeric enzyme has also been observed in cyanide-resistant alternative oxidase from the mitochondria of the voodoo lily (*Amorphophallus kiusianus*), soybean, and mung bean (*Vigna radiate*; Umbach and Siedow, 1993), and their oxidation reduction states are responsible for their activities. In animals, the active AChE was found in monomeric, disulfide-linked dimeric, and tetrameric forms, depending upon the animal species. For exam-

ple, Sipunculida (Talesa et al., 1993) and Insecta (Gnagey et al., 1987; Toutant 1989) produce only the disulfide-linked dimeric form. The oxidation reduction states of the disulfide bond between the subunits in the maize AChE dimeric form could dominate an enzymatic activity, similar to the cyanide-resistant alternative oxidase. However, we could not determine which form was responsible for the AChE activity in this study because of the small yield of enzyme that was purified.

In general, cholinester-hydrolyzing enzymes are classified into the AChE and BChE classes, depending on their substrate and inhibitor specificity. AChE preferably hydrolyzes ACh or propionylcholine more readily than butyrylcholine. In contrast, BChE hydrolyzes butyrylcholine more quickly. The purified maize AChE displayed relatively high affinity to PpSCh and was followed in affinity by ASCh among the thiocholinesters employed, while BSCh was apparently a poor substrate. This indicates that the maize enzyme was characterized as an AChE, not a BChE. In addition, neostigmine bromide is a specific AChE inhibitory reagent that binds to the catalytic site of the animal AChE protein via a covalent carbamyl bond (Standaert, 1990), but its inhibition mode is nearly competitive probably because the enzyme and inhibitor initially forms reversible complex, and then the binding mode slowly changes to covalent (Kishibayashi et al., 1994). Furthermore, the neostigmine bromide was previously found to inhibit the ACh-hydrolyzing activity in the plant tissues, as well as animal AChE (Momonoki, 1992). Neostigmine bromide inhibited the hydrolytic activity of the purified maize AChE in a typically competitive manner. This means that neostigmine bromide competes with the substrates for binding to the active site of the maize AChE, in the same manner as it inhibited the animal AChE. These findings strongly indicate that the maize AChE has an active site similar to that of the animal AChE protein. The amino acid sequences of the maize and electric eel AChE precursors, however, shared extremely low homology with scores of 11.7% on ClustalW analysis. Computer-assisted protein family analysis using the Pfam database server implied that the maize AChE contains a consensus sequence of the lipase GDSL family at residues 41 to 373, and this region possesses a characteristic consensus Gly-Asp-Ser-Leu-Ser sequence. Most proteins with this consensus sequence are involved in removal of the acyl chain from esters or lipids by hydrolysis. The catalytic Ser/Asp (Glu)/His triads of the enzymes belonging to lipase GDSL family were characterized in some of these proteins, and the sequences surrounding the consensus residues are well conserved (Upton and Buckley, 1995). Therefore, the putative catalytic triad for the maize AChE, Ser-47/Asp-185/His-360, can be deduced by superimposing the sequences on those of the lipase GDSL family enzymes (Fig. 6). Although the animal AChE also has a catalytic Ser/Asp (Glu)/His triad (Shafferman et al., 1992), it possesses a distinct carboxylesterase

Maize-AChE	41	VFNFGD <b>NS</b> SDT	(120)	171	YFSQALYTFD <b>IG</b> QNDITS	(165)	RVSWDGVH <b>FT</b> EAANKV <b>FD</b> QI	373
Digitalis-LAE	36	IFNFGD <b>NS</b> SDT	(117)	163	IFRKS <b>LY</b> TL <b>YI</b> Q <b>ND</b> FTG	(164)	YVSWD <b>GI</b> HATEAANKV <b>HA</b> AI	364
Rape-APG	126	VFFF <b>GD</b> S <b>IF</b> FD <b>T</b>	(121)	257	IVSK <b>GA</b> IVV <b>AG</b> S <b>ND</b> LIY	(147)	YLFWD <b>GL</b> H <b>PS</b> Q <b>RA</b> Y <b>EI</b> SN <b>RK</b> L	441
Rape-MyAP	36	LF <b>TF</b> FGD <b>SN</b> FDA	(100)	146	YITK <b>SL</b> FM <b>IS</b> IG <b>ME</b> D <b>Y</b> N	(137)	YLF <b>FD</b> GR <b>HN</b> SE <b>KA</b> Q <b>EQ</b> FA <b>HL</b> L	320
Rabbit-AdrAB-B (A)	394	IG <b>AM</b> GD <b>SL</b> T <b>AG</b>	(96)	500	QED <b>WK</b> II <b>TV</b> FI <b>GG</b> ND <b>LC</b> D	(131)	FF <b>AP</b> DC <b>HF</b> FS <b>SK</b> A <b>HA</b> HA <b>AS</b> AL	668
Rabbit-AdrAB-B (B)	741	VA <b>AL</b> GD <b>SL</b> T <b>AG</b>	(96)	847	QED <b>WK</b> VIT <b>VQ</b> IG <b>AS</b> DL <b>CD</b>	(131)	FF <b>AP</b> DC <b>VP</b> NP <b>QK</b> FS <b>QL</b> SR <b>AL</b>	1015
Rabbit-AdrAB-B (C)	1097	VA <b>AL</b> GD <b>SL</b> T <b>LA</b>	(91)	1198	E <b>KD</b> W <b>KL</b> V <b>TL</b> F <b>V</b> GG <b>ND</b> L <b>CH</b>	(128)	FF <b>S</b> DD <b>CF</b> H <b>S</b> ER <b>GHA</b> EM <b>AIA</b> L	1363
Ruminococcus-AXE	46	IM <b>PL</b> GD <b>S</b> I <b>TY</b> G	(83)	139	K <b>YS</b> PD <b>II</b> LL <b>Q</b> IG <b>T</b> ND <b>VS</b> N	(80)	DL <b>AD</b> G-V <b>HP</b> NA <b>GG</b> Y <b>EK</b> MG <b>KY</b> W	256
Neocallimastix-AXE	65	IM <b>PM</b> GD <b>S</b> I <b>TF</b> G	(80)	155	L <b>AK</b> PD <b>II</b> LL <b>II</b> GT <b>ND</b> MS <b>G</b>	(79)	DM <b>SS</b> DK <b>VH</b> PS <b>GS</b> GY <b>KK</b> MG <b>DY</b> F	271
Agrobacterium-ArE	5	V <b>LC</b> FGD <b>SL</b> T <b>WG</b>	(63)	78	H <b>AP</b> LD <b>LV</b> II <b>ML</b> GT <b>ND</b> L <b>KP</b>	(89)	T <b>TP</b> V <b>DG</b> V <b>HL</b> DA <b>ENT</b> RA <b>IG</b> RL	204
Vibrio-HLT	147	V <b>VA</b> LG <b>D</b> SL <b>S</b> DT	(102)	259	E <b>VK</b> AD <b>Y</b> AE <b>AL</b> IR <b>L</b> T <b>D</b> GA	(110)	F <b>V</b> FW <b>DV</b> TH <b>PT</b> T <b>ATH</b> RY <b>V</b> AE <b>KM</b>	406
Aeromonas-GCAT	28	I <b>VM</b> FG <b>D</b> SL <b>S</b> DT	(115)	153	I <b>SD</b> A <b>AN</b> RM <b>V</b> L <b>NG</b> AK <b>E</b> ILL	(132)	K <b>M</b> FW <b>DQ</b> VH <b>PT</b> TV <b>V</b> HA <b>AL</b> SE <b>PA</b>	322
Yeast-IAH	6	F <b>LL</b> FGD <b>S</b> I <b>TE</b> F	(54)	70	E <b>SN</b> IV <b>MAT</b> I <b>FL</b> GA <b>ND</b> ACS	(96)	Q <b>LL</b> T <b>D</b> GL <b>H</b> FS <b>GK</b> GY <b>KI</b> F <b>H</b> DEL	203
Human-AOAH	257	I <b>ILL</b> GD <b>S</b> AG <b>AH</b>	(93)	360	L <b>DY</b> PA <b>IV</b> I <b>Y</b> AM <b>I</b> GN <b>D</b> VCS	(146)	I <b>EP</b> V <b>DG</b> F <b>HP</b> NE <b>V</b> ALL <b>L</b> L <b>AD</b> HF	543
Aspergillus-RGAE	20	V <b>YL</b> LAG <b>D</b> S <b>IM</b> AK	(60)	90	H <b>ND</b> GG <b>S</b> LS <b>T</b> DN <b>GR</b> T <b>D</b> CS <b>G</b>	(98)	Y <b>FP</b> ID <b>H</b> TH <b>TS</b> P <b>AGA</b> EV <b>V</b> AA <b>EF</b>	225
E. coli-TESA	30	L <b>LL</b> LGD <b>S</b> LS <b>AG</b>	(46)	86	Q <b>HQ</b> PR <b>W</b> VL <b>VEL</b> GG <b>ND</b> GL <b>R</b>	(76)	--- <b>DD</b> GI <b>HP</b> NR <b>DA</b> Q <b>PP</b> I <b>AD</b> WM	196
Vibrio-ArE	23	L <b>LV</b> LGD <b>S</b> LS <b>AG</b>	(49)	82	Q <b>HT</b> PD <b>L</b> VL <b>L</b> IEL <b>GA</b> ND <b>GL</b> R	(76)	--- <b>DD</b> GL <b>HP</b> K <b>PE</b> A <b>QP</b> W <b>IA</b> EF <b>V</b>	192

**Figure 6.** Alignment of the amino acid sequences of maize AChE and selected members of the GDSL lipase family. The alignment was generated by a ClustalX analysis. Dashes indicate gaps introduced to improve the alignment. Numbers in brackets indicate intervening amino acid numbers, and the numbers without brackets indicate the positions in the sequences. The putative catalytic triad Ser/Asp (Glu)/His residues in the GDSL family enzymes, which were presumed previously (reference in Upton and Buckley, 1995), are indicated by arrowheads. The abbreviations and accession numbers of the cDNA nucleotide sequences used for this analysis are follows: Maize-AChE, *Zea mays* AChE (determined in this study); Digitalis-LAE, *Digitalis lanata* lanatoside 15'-*O*-acetyltransferase (AJ011567); Rape-APG, *Brassica napus* anter-specific Pro-rich protein APG (P40603); Rape-MyAP, *Brassica napus* myrosinase-associated protein (U39289); Rabbit-AdrAB-B, *Oryctolagus cuniculus* phospholipase (Z12841); Ruminococcus-AXE, *Ruminococcus flavefaciens* acetyl xylan esterase (AJ238716); Neocallimastix-AXE, *Neocallimastix patriciarum* acetyl xylan esterase bnIII (U66253); Agrobacterium-ArE, *Agrobacterium radiobacter* arylesterase (AF044683); Vibrio-HLT, *Vibrio parahaemolyticus* thermolabile hemolysin (M36437); Aeromonas-GCAT, *Aeromonas hydrophila* glycerophospholipid-cholesterol acyltransferase (X07279); Yeast-IAH, *Saccharomyces cerevisiae* isoamyl acetate hydrolytic enzyme (X92662); Human-AOAH, *Homo sapiens* acyloxyacyl hydrolase (BC025698); Aspergillus-RGAE, *Aspergillus aculeatus* rhamnogalacturon acetyltransferase (X89714); *E. coli*-TESA, *Escherichia coli* acyl-coA thioesterase I (L06182); Vibrio-ArE, *Vibrio mimicus* arylesterase (X71116).

consensus domain, but not the lipase GDSL consensus, based on Pfam protein family analysis. Thus, the maize AChE could not belong to the well-known AChE family, which is distributed throughout the animal kingdom. Based on in silico screening against the plant gene database, the genes homologous to maize AChE are widely distributed in higher plants (Fig. 5). On the other hand, a tBLASTn search of the maize AChE sequence against animal, fungi, and bacterial genomic sequence databases revealed no homologs. Therefore, we appear to have discovered a novel AChE family that is specifically distributed in the plant kingdom and that might be evolved from a different family of ancestor protein of the well-known AChE class. According to a report by Fluck and Jaffe (1975), the cholinesterase purified from eggplant (*Solanum melongena*), but not that from maize, was inhibited by superoptimal substrate concentration. Because the inhibition by the excess of the substrate was regarded as characteristic and common feature of the animal AChE, they had concluded that the maize cholinesterase was not real AChE, despite the enzyme specifically hydrolyzed ASCh among thiocholinesters employed. In this study, we also found that the maize AChE purified did not exhibit the inhibition by superoptimal substrate (data not shown). Among the animal AChE, the inhibitory effects of the excess of the substrate molecules on the activity might be variable depending on the source organisms and tissues, probably due to the structural difference around the active

site (Tōgu, 2001). Indeed, an AChE, whose activity is not affected under the superoptimal substrate condition, was recently found from an oriental fruit fly *Bactrocera dorsalis* (Hsiao et al., 2004). Therefore, the discrepancy of the inhibition at high substrate concentration may be reflected by such structural difference also among the plant enzymes. As an alternative possibility, two genes in the published Arabidopsis database were suggested to have similarity with animal AChE (Fluck et al., 2000), although their gene products have not yet been recognized from the plant tissues. Thereby, multiple classes of enzymes associating with ACh-hydrolyzing activity could be distributed in the plant kingdom, which might result in the difference of the substrate inhibition manner as observed in the enzymes purified from eggplant and maize. On the other hand, the maize AChE showed a low affinity for the substrate, compared with the electric eel AChE. In addition, the maize AChE required a high concentration of neostigmine bromide for complete inhibition and displayed a low affinity to this reagent, compared with the electric eel AChE. ACh has been recognized even in evolutionally primitive organisms, such as bacteria (Horiuchi et al., 2003); thus, its function as neurotransmitter might be specialized in the animal kingdom. The animal AChE is known to be one of the fastest enzymes. In animal nerve systems, AChE degrades the neurotransmitter, ACh, to terminate signaling by preventing adsorption of ACh to the receptor. The quick reactivity of AChE could be essential to rapid

control of synaptic activity, so animal AChE may have acquired a high affinity to the substrate and hydrolytic efficiency through molecular evolution (Shen et al., 2002). The difference in the affinity to the substrate between the plant and animal AChE, thereby, might be dominantly related to the different biological roles of the enzyme in each kingdom, rather than the loss of the affinity due to partial inactivation of the maize AChE during purification.

The computer-assisted cellular sorting prediction program TargetP presumed that the maize AChE is targeted to the secretory pathway via the endoplasmic reticulum. Furthermore, the SOSUI program (<http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>), which discriminates between membrane and soluble proteins, showed that the maize AChE does not contain any likely transmembrane helical regions, which are features of proteins that associate with lipid bilayers of the cell membrane. Histochemical analysis of maize seedlings showed that the AChE activities were localized at peripheral regions of the coleoptile, coleoptile node, and mesocotyl cells (Momonoki, 1997). According to Fluck and Jaffe (1974b), most of the AChE activities in the maize root were associated with the cell wall materials, based on AChE assays of the cell organelle fractions. Moreover, they demonstrated that a salt solution could effectively solubilize the AChE activities from cell wall materials. In this study, we used a high salt buffer containing 4% ammonium sulfate for the AChE extraction from the maize seedling homogenates, while a low salt buffer without 4% ammonium sulfate yielded relatively low AChE activity from the homogenate (data not shown). Therefore, the maize AChE, which is glycosylated through the secretory pathway, could be localized in the cell walls via a probable interaction between the sugar chain on the molecule and polysaccharides in the cell wall materials. In a previous report (Momonoki, 1997), it was speculated that AChE is localized at the plasmodesmatal cell-to-cell interface and that it plays a role in regulation of the plasmodesmatal channel as a constituent of the ACh-mediated system. However, it was speculated that the maize AChE might be localized at the cell wall, based on findings in this study. Thus, we can suggest improvements to the model of the ACh-AChR-AChE systems proposed in the original report (Momonoki, 1997). In particular, the system might be localized to the extracellular region around the plasmodesmatal channel and might conduct cell-to-cell trafficking by a channel gating regulation. In plant tissues, the two adjoining cells are interconnected via plasmodesmata, which allow the trafficking of low-molecular-mass materials across the cell wall between two cells. According to a recent model (Lucas, 1995), transport of these substances could be regulated by the opening or/and closing of conductive channels to prevent infection by pathogens and to selectively control trafficking through the plasmodesmata. Moreover, it has been speculated that morphoregulatory proteins around the plasmodesmata could be in-

involved in channel regulation (Cassab, 1998). The ACh-mediated system might regulate the opening and/or closing of channels by interaction with morphoregulatory proteins at the cell wall matrix surrounding the plasmodesmata.

Recently, the ACh molecule has been definitively identified not only in animal tissues, but also in fungi and plant tissues. Furthermore, recent reports suggest that the plant ACh is involved in the regulation of water homeostasis and photosynthesis (Wessler et al., 2001). However, nothing had been known about ACh-synthesizing enzyme(s), AChRs, and ACh-hydrolyzing enzyme(s) in the plant kingdom; thus, knowledge of plant ACh has remained primitive. According to Ballal et al. (1993), indol-3-ACh, the choline conjugate of the IAA, stimulates the elongation of the pea (*Pisum sativum*) stem segments. More recently, Fluck et al. (2000) suggested a model as the pea cholinesterase might release the free IAA from the conjugates, but they failed to obtain the direct molecule evidence. Therefore, the discovery of an AChE gene here is the first direct evidence, to our knowledge, of the AChE molecule in the plant kingdom, and this will surely provide us with a better understanding of the ACh-AChR-AChE systems in this kingdom. Further characterization of its precise structure, e.g. via crystallography, will be required to clarify the relationship between the molecular conformation and the catalytic mechanism, which still remains unclear. In addition, the expression patterns and cellular localization of the AChE gene products and identification of additional ACh-related proteins would be required to clarify the precise role of the ACh-AChR-AChE systems in the plant kingdom.

## MATERIALS AND METHODS

### Plant Materials

Kernels of *Zea mays* L. cv Silver Honey Bantam sweet corn were rinsed with 10% sodium hypochlorite and then soaked overnight in running water. After stabilizing with sodium hypochlorite (1.2% of the available chloride) and rinsing with distilled water, the kernels were placed in a plastic container (diameter, 12.5 cm; height, 19.5 cm) and germinated in rolled moist paper towels for 5 d in a darkroom at 28°C and 80% humidity. The seedlings' roots were then harvested and stored at -30°C until use for AChE purification.

### Purification of AChE from Maize Seedlings

Two hundred grams of 5-d-old etiolated maize seedlings were homogenized in a 4-fold volume (v/w) of extraction buffer consisting of 10 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, and 4% ammonium sulfate. Subsequent purification steps were carried out at 4°C or on ice. The homogenate was left for 1 h in the dark, and then the supernatant was collected by centrifugation at 6,000g for 15 min and stored at 4°C. The precipitate was resuspended in a 3-fold volume (v/w) of the extraction buffer, along with an equal weight of quartz pieces (size 0.3 to 0.5 mm, 30 to 50 mesh; Kishida Chemical Co., Osaka), and was stirred for 1 h. This procedure was repeated twice. The extracts were combined and brought to 80% saturation with ammonium sulfate and left overnight. The resulting precipitate was collected by centrifugation at 15,000g for 15 min, resuspended in 3 mL of phosphate-buffered saline, pH 7.4, and then dialyzed against the same buffer. The precipitate that formed during dialysis was removed by centrifugation,

and the supernatant was stored at  $-30^{\circ}\text{C}$  until use for the chromatographic purification procedure.

### AChE Assay

One hundred microliters of the AChE preparation were added to 150  $\mu\text{L}$  of distilled water or neostigmine bromide (8 mM) in 100 mM sodium phosphate buffer, pH 7.0, as a control. After preincubation for 10 min at  $30^{\circ}\text{C}$ , 250  $\mu\text{L}$  of 12.5 mM ASCh chloride in 100 mM sodium phosphate buffer, pH 7.0, was added to the vials, and incubation was continued for 120 min at  $30^{\circ}\text{C}$ . After incubation, 300  $\mu\text{L}$  of solution was transferred to a new vial, and 1,425  $\mu\text{L}$  of 100 mM sodium phosphate buffer, pH 7.0, and 75  $\mu\text{L}$  of DTNB in 100 mM sodium phosphate, pH 7.0, were added to the vial. The  $A_{412}$  was read after 1 min. All experiments were adjusted using the control described above. One enzyme unit was defined as 1  $\mu\text{mol}$  of thiol group appearance/min.

Substrate specificities of the maize and electric eel AChEs were examined by using PpSCh chloride and BSCh chloride as substrates instead of ASCh chloride, following the same procedure described above. To determine  $K_i$  values for neostigmine bromide inhibition of ASCh and PpSCh hydrolysis by the maize and electric eel AChE, the parameters for Dixon plots ( $1/v$  and  $[I]$ ) were measured by the ASCh and PpSCh hydrolysis assay according to the same procedure described above. Experiments were carried out in the presence of several sets of concentrations of neostigmine bromide and the substrates, ASCh and PpSCh. The  $K_i$  values were calculated according to the method of Dixon (1953). All measurements were repeated three times, and the results represent the arithmetic means.

### PAGE Analysis

SDS-PAGE was performed as described by Laemmli (1970) using a 12.5% or 15% gel. Phosphorylase b (97.4 kD), bovine serum albumin (66.3 kD), aldolase (42.4 kD), carbonic anhydrase (30.0 kD), and soybean trypsin inhibitor (20.1 kD) were used for protein standards. Native PAGE, using a slab minigel (10.8%), was performed according to the procedure described by Davis (1964). The activity staining solution was adjusted to 6 mM ASCh and 3 mM DTNB in 100 mM sodium phosphate buffer, pH 7.0. The native-PAGE gel resolved AChE preparation was soaked into the activity staining solution at room temperature, until the colored band was visualized.

### Analytical Gel Filtration for Estimation of Molecular Mass

The sample was applied to Superdex 200 HR10/30 column equilibrated with phosphate buffer, pH 7.0, containing 0.15 M NaCl and 10% glycerol. Molecular size markers were  $\beta$ -amylase (200 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD), carbonic anhydrase (29 kD), and cytochrome C (12.4 kD). Molecular size was estimated from the mean of three experiments from a plot of the log of molecular mass versus elution volume.

### Partial Amino Acid Sequence Analysis of the AChE Protein

The N-terminal amino acid sequences of the AChE protein and its derivative polypeptides were determined by the direct protein sequencing method described by Hirano and Watanabe (1990). The derivative polypeptides of the AChE protein were prepared by proteolytic digestion with *Staphylococcus aureus* V8 protease (Wako Pure Chemical, Osaka) during electrophoresis according to the method described by Cleveland et al. (1977). The AChE protein separated by SDS-PAGE was electroblotted onto a polyvinylidene difluoride membrane using a semidry blotting apparatus (Nippon Eido, Tokyo). After the membrane was stained, visible bands were cut out. The N-terminal amino acid sequence of the parent AChE protein and its derivative polypeptides were determined using a pulsed-liquid phase sequencer (model 477A; PE-Applied Biosystems) equipped with online HPLC (model 120A; PE-Applied Biosystems).

### Determination of the Nucleotide Sequence of the cDNA Encoding AChE

Frozen 5-d-old seedlings were ground into powder with an adequate volume of liquid nitrogen. Total RNA was isolated from the seedling powder

(3 g) using the RNeasy plant mini kit (Qiagen, KJ Venlo, The Netherlands) according to the manufacturer's instructions. The cDNAs were synthesized from the total RNA preparation by 3'- and 5'-RACE using the SMART RACE cDNA amplification kit (MD Biosciences, CLONTECH, Palo Alto, CA). Amplification of the RACE fragments corresponding to the AChE gene was performed by PCR using gene-specific primers, Mmn-2 for the 5'-RACE and Sgn-1 for the 3'-RACE. PCR was performed in a 50- $\mu\text{L}$  reaction mixture containing 2.5  $\mu\text{L}$  of cDNA solution, 0.5  $\mu\text{M}$  of each primer, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM each deoxynucleotide triphosphate, 5% dimethylsulfoxide, and 1.25 units of Gene Taq Polymerase (Nippon Gene, Osaka). The PCR reaction was carried out for 35 cycles consisting of 1 min at  $94^{\circ}\text{C}$ , annealing for 1 min at  $57^{\circ}\text{C}$ , and 2 min at  $72^{\circ}\text{C}$  using a Gene-Amp automated thermal cycler (model 9700; PE-Applied Biosystems). The PCR products were separated by agarose gel electrophoresis and then extracted from the gels using an EasyTrap DNA extraction kit (Takara, Ohtsu, Japan). The purified fragments were subcloned into a pT7Blue T-vector (Novagen, Madison, WI) according to the manufacturer's instructions. The purified DNA was sequenced on both strands using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE-Applied Biosystems). The primers indicated in Table III and the attached primers in the SMART RACE cDNA amplification kit were used for cycle sequencing. If necessary, additional primers for the cycle sequencing were designed based on the determined sequence. MacVector nucleotide and protein analysis software (Oxford Molecular) was utilized for the sequence analysis.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank data libraries under accession number AB093208.

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