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Identification and Biochemical Characterization of Plant Acylamino Acid–Releasing Enzyme

Yasuo Yamauchi*, Yukinori Ejiri, Yasuyuki Toyoda and Kiyoshi Tanaka
Laboratory of Plant Biotechnology, Faculty of Agriculture, Tottori University, Koyama, Tottori 680-8553

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Plant acylamino acid-releasing enzyme (AARE) catalyzing the *N*-terminal hydrolysis of N^{α}-acylpeptides to release N^{α}-acylated amino acids, was biochemically characterized using recombinant and native AAREs. A cDNA encoding a deduced *Arabidopsis thaliana* AARE (AtAARE) was cloned and sequenced. The deduced amino acid sequence encoded a 764 amino acid protein of 83.9 kDa, which was 31.8% identical with that of rat AARE. In particular, the proposed catalytic residues (Ser, Asp, and His) of AARE, called the "catalytic triad residues, " were completely conserved. Recombinant AtAARE was expressed in *Escherichia coli* and confirmed to be a functional AARE. Native AAREs were prepared from *A. thaliana* and cucumber (*Cucumis sativus*, L.) plants. Both native AAREs were tetrameric proteins of 350 kDa comprising four subunits of 82 kDa, and showed typical enzymological properties of other AAREs, *i.e.* sensitivity to diisopropyl fluorophosphate, an optimum pH of around 7.0, and an optimum temperature of 37°C. Both the native and recombinant AAREs were immunochemically homologous. Intracelluar fractionation analysis showed that the AARE was mainly present in the stroma of chloroplasts. Native AARE degraded the glycated ribulose-1,5-bisphoshate carboxylase/oxygenase protein but not the native protein. Thus, plant AARE might be involved in not only catalysis of the N-terminal hydrolysis of N^{α}-acylpeptides but also the elimination of glycated proteins.

Key words: acylamino acid-releasing enzyme, *Arabidopsis thaliana*, *Cucumis sativus*, glycation, protein degradation.

Abbreviations: AARE, acylamino acid-releasing enzyme; Ac, acetyl; AGE, advanced glycation end-product; AtAARE, *Arabidopsis thaliana* AARE; cAARE, cucumber AARE; IPTG, isopropyl β-D-thiogalactopyranoside; 2-ME, 2-mercaptoethanol; PB, phosphate buffer; pNA, *p*-nitroanilide; rAtAARE, recombinant *Arabidopsis thaliana* AARE; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

The acylamino acid-releasing enzyme (AARE) catalyzes the N-terminal hydrolysis of N^{α}-acylpeptides, which releases N^{α}-acylated amino acids (*1*), and it has been well characterized in mammals. The enzyme is thought to be involved in the turnover and destabilization of N^{α}-acetylated proteins and peptides, because more than half of the intracellular proteins are N^{α}-acetylated in mammals (*2*, *3*), and N^{α}-acylation may affect the biological functions of proteins by stabilizing them or influencing their degradation pathways (*4*). Recently, one possible function of AARE was proposed, *i.e.* AARE, first identified as an oxidized protein hydrolase, might play an important role in destroying glycated and oxidatively damaged proteins in living cells (*5*, *6*). Interestingly, AARE shows not only exopeptidase-like activity but also chymotrypsin-like endopeptidase activity toward oxidized proteins (*7*).

In plants, a high percentage of proteins is assumed to be N^{α}-acylated. For example, the N-terminal sequence of more than 70% of rice shoot proteins could not be determined by protein sequencing, which was explained as being due to N^{α}-acylation (*8*). Moreover, the glycation of a protein might easily occur under intracellular conditions in plants (*9*). A plant AARE has not been characterized so far, however, AARE will be widely distributed in plants, because homologous genes have been identified in several plants, and might be involved in the turnover of N^{α}-acylated and chemically damaged proteins like mammalian AAREs.

In this study, we cloned the deduced plant AARE gene in *Arabidopsis thaliana* and performed biochemical characterization of the encoded protein. Additionally, native AAREs were prepared from *A. thaliana* and cucumber, and detailed enzymological properties including the ability of degrading glycated proteins were also examined.

MATERIALS AND METHODS

Materials—The KOD DNA polymerase and restriction enzymes were purchased from Toyobo (Tokyo). The synthesis of DNA primers was performed by the custom service of Espec Oligo Service (Tsukuba, Ibaraki). Anti-advanced glycation end product (AGE) monoclonal antibody (*6*D*12*) was purchased from Dojindo Laboratories (Kumamoto). Oligopeptides were generous gifts from Dr. N. Mori of the Faculty of Agriculture, Tottori University. All other reagents were purchased from Wako Chemical (Osaka).

Cucumber seeds (*Cucumis sativus* L. *suyo*) purchased from Takii Seed (Kyoto) were germinated and grown in

*To whom correspondence should be addressed. Fax: +81-0-857-31-6702, E-mail: yamauchi@muses.tottori-u.ac.jp

the field. *Arabidopsis thaliana* ecotype Columbia was grown in a growth chamber at 23°C (light 12 h/dark 12 h) with Hyponex. Harvested materials were frozen in liquid N_2 and stored at –80°C until use.

Cloning, Sequencing and Expression of Arabidopsis AARE (AtAARE)—Total RNA was prepared from 1.5 g of 6-week-old *A. thaliana* shoots by the acid-guanidine-phenol chloroform method. After isolation of mRNA with Oligotex dT (Takara Shuzo, Otsu, Shiga), cDNA was synthesized with a First Strand Synthesis Kit (Amersham Pharmacia Biotech, Sweden). The open reading frame encoding a putative AARE was amplified by the polymerase chain reaction method using two primers (upper primer, 5′-ATG GAT TCT TCT GGA ACT GAT TCG GC-3′; lower primer, 5′-TCA CAG CTT GCA GTA CTT GTT GAA CC-3′) with cDNA as a template. Amplification of the gene by polymerase chain reaction was carried out at 96°C for 1 min, 48°C for 1 min, and 74°C for 2 min, for 30 cycles, using KOD DNA polymerase. The amplified gene was kinated with a Blunting Kination Kit (Takara Shuzo) and then inserted in pQE32 (Qiagen, Hilden, Germany) cut with *Sma*I. To confirm the cloned gene was that of AtAARE, DNA sequencing was performed with a DNA sequencer (ABI Prism 377; Applied Biosystems, CA). Recombinant AtAARE (rAtAARE) was expressed using a QIAexpressionist system in the host *E. coli* M15 according to the manufacturer's instructions (Qiagen). After incubation of the transformant cells in LB medium containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml) with shaking at 37°C until the A_{600} reached 0.6–1.0, the induction of rAtAARE was carried out by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and shaking for 3 h at 25°C.

Amino Acid Sequencing of rAtAARE—Two μg of rAtAARE purified by electrophoresis was dissolved in 1 ml of 70 % formic acid containing 1 mg of CNBr, and then stood overnight in the dark. After evaporation in a SpeedVac Concentrator (model A160; SAVANT Instruments, NY), the cleaved peptides were dissolved in 50 μl of SDS sample buffer and then subjected to Tricine/SDS-PAGE on a 15% polyacrylamide gel (*10*). After electroblotting onto a polyvinylidene difluoride membrane, the peptides were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 45% MeOH and 10% AcOH, and portions of the major bands were excised and sequenced with an automated pulsed liquid protein sequencer (model 491; Applied Biosystems).

Point Mutation of the AtAARE Gene—In vitro site-directed mutagenesis was performed with a QuickChange® XL Site-directed Mutagenesis Kit (Stratagene, CA) according to the instruction manual. The mutagenesis primers used were, "GTG CTA GGT GGT tCT CAT GGT GGG TTT C" and "GAA ACC CAC CAT GAG aAC CAC CTA GCA C" for the S618A mutation, "TTG GGA ACT AAG aAT CTC CGT GTT CCC" and "GGG AAC ACG GAG ATt CTT AGT TCC CAA" for the D707N mutation, and "CCC AAT GAC AAT gcT CCC TTA GAT AGA CC" and "GGT CTA TCT AAG GGA gcA TTG TCA TTG GG" for the H739A mutation, respectively. The mutations were confirmed by DNA sequencing.

Enzyme Activities—For the standard assay, we used a reaction mixture comprising enzyme solution and 1 mM acetyl (Ac)-Ala-*p*-nitroanilide (pNa; Sigma, St. Louis, MO) in 50 mM Hepes-KOH, pH 7.0, in a total volume of 1 ml (the reaction mixture contained at least 0.1 mM 2-ME as a stabilizer). After incubation at 37°C, the reaction was stopped by the addition of 0.5 ml of 30% (v/v) acetic acid and then the absorbance at 410 nm was measured. For assaying with an acylated oligopeptide as a substrate, the liberated amino acid was measured by means of the ninhydrin reaction (*11*). Determination of the hydrolysed products of oligopeptides with AARE was carried out by HPLC. The amino acids were *N*-hydroxysuccinyl-6-aminoquinolyl carbanized with an AccQ Fluor Reagent Kit according to manufacturers' instructions (Waters, MA), and the derivatives were analyzed by reversed-phase HPLC on a NovaPac C18 column (Waters). The column was equilibrated with 140 mM sodium acetate, pH 5.8, adjusted with triethylamine. After 10 μl of a sample had been injected, a 0–50% acetonitrile gradient was used for elution (Total, 40 ml). The flow rate was 1.0 ml/min and the elution time of each derivative was determined with a fluorescence detector (Ex 250 nm, Em 395 nm; FS8010, Hitachi).

Purification of Native AAREs from Plants—Cucumber leaves (300 g) or *Arabidopsis* shoots (30 g) were homogenized with 1% (w/w) polyvinylpolypyrrolidone and 5 volumes of 50 mM potassium-phosphate buffer (K-PB), pH 7.0, containing 2 mM 2-mercaptoethanol (2-ME) and 1 mM EDTA. After centrifugation at 20,000 g for 20 min, the supernatant was fractionated by the addition of solid ammonium sulfate. The 45–60% ammonium sulfate saturation precipitate was dissolved in a minimum volume of 25 mM K-PB, pH 7.0, containing 1 mM 2-ME, and then dialyzed against the same buffer. The dialysate was applied to a DEAE-Toyopearl (4 × 20 cm; Tosoh, Tokyo) column equilibrated with 25 mM K-PB, pH 7.0, containing 1 mM 2-ME, and then a linear gradient of 0 to 0.3 M NaCl was applied to elute the enzyme. After the active fractions had been combined, a 1/3 volume of 80% saturated ammonium sulfate in 50 mM K-PB, pH 7.0, containing 1 mM 2-ME and 0.1 mM EDTA was added to give a final concentration of 20%. The solution was then applied to a Phenyl-Sepharose (1.5 × 8 cm; Amersham Biosciences, Tokyo) column equilibrated with 20% ammonium sulfate-saturated 50 mM K-PB, pH 7.0, containing 1 mM 2-ME and 0.1 mM EDTA, and then eluted with a linear gradient of 20 to 0% ammonium sulfate. The active fractions were combined and then dialyzed against 10 mM sodium phosphate buffer (Na-PB), pH 6.8, containing 1 mM 2-ME. The dialysate was applied to a hydroxyapatite (1.0 × 5 cm; Bio-Rad Laboratories, CA) column and then eluted with a linear gradient of 0 to 0.2 M Na-PB, pH 6.8. In the case of cucumber AARE (cAARE), the active fractions were concentrated with a Vivapore 10 (7,500 MWCO; Vivascience, UK), and then further purified on a gel filtration (HW-55F, 1.5 × 60 cm; Tosoh) column equilibrated with 25 mM K-PB, pH 7.0, containing 1 mM 2-ME and 0.1 M NaCl). The purified AAREs were concentrated and stored at –20°C in the presence of 40% glycerol.

Characterization of AARE—In order to determination the effects of inhibitors, AARE was preincubated with each inhibitor in the reaction mixture without a substrate for 30 min at room temperature. The substrate was then added to the mixture and activity was measured by the standard method. SDS-PAGE was performed

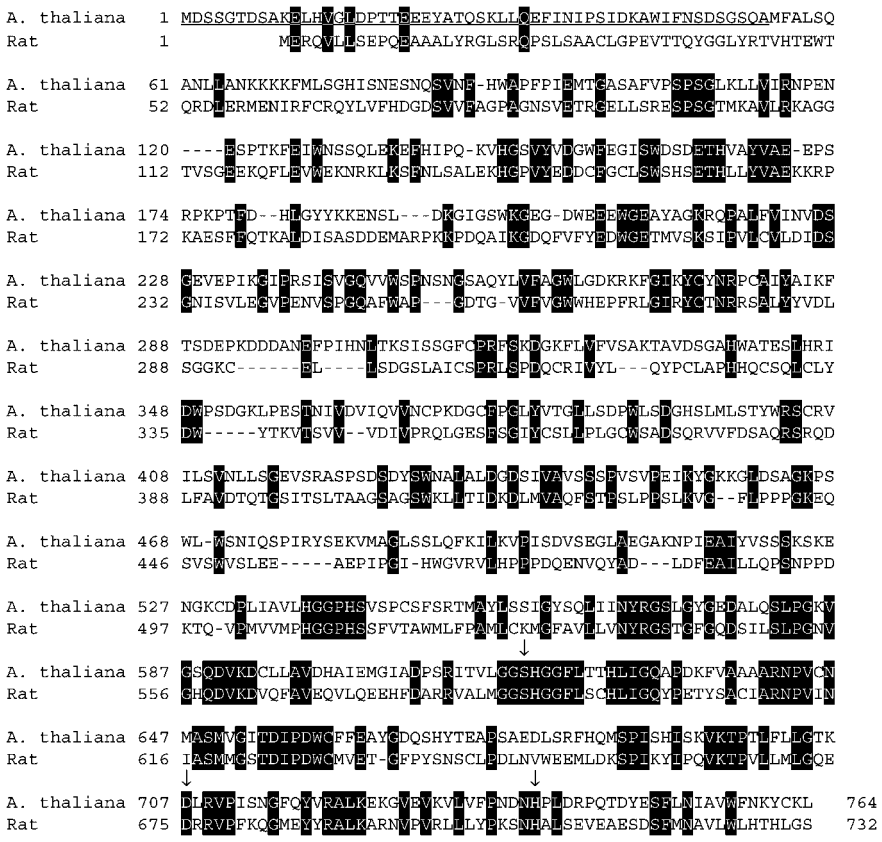

Fig. 1. **Alignment of the sequences of AtAARE and rat AARE.** Sequence alignment was performed by the Lipman-Pearson method (*24*) using the Genetyx-SV/RC program (ver 11.0; Software Development, Tokyo). The sequences have been aligned with dashes indicating gaps. Conserved residues in two enzymes are shaded. Arrows indicate putative active residues (Ser-618, Asp-707, and His-739 of AtAARE). A putative signal sequence is underlined. Accession numbers of AtAARE and rat AARE are BAC76411 and P13676, respectively.

on a 10% (w/v) acrylamide gel by the method of Laemmli (*12*). Gels were stained for protein with Coomassie Brilliant Blue R-250. Western blotting was performed according to the manufacturers' instructions after transblotting to a polyvinylidene difluoride membrane (Atto, Tokyo). Anti-rAtAARE antiserum prepared by the method described previously (*9*) was used as the primary antiserum (1:500 dilution). Alkaline phosphatase-conjugated anti-mouse IgG antibodies (StressGen Biotechnologies, Canada) were used as the secondary antibodies, and signals were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoryl phosphate. Isoelectric focusing was performed on a 6% (w/v) acrylamide gel containing 2% Ampholine (pH 4.0–6.5; Amersham Biosciences), 2 mM 2-ME and 2.5% (v/v) glycerol. Forty mM glutamic acid and 0.2 M histidine were used as the cathode and anode buffers, respectively. After electrophoresis at 600V for 3 h, the pH and AARE activity of gel slices (every 0.5cm) were measured. Protein was determined by the method of Lowry *et al*. with bovine serum albumin as the standard (*13*).

Subcellular Fractionation—Subcellular fractionation was carried out by conventional sucrose density gradient centrifugation. Cucumber cotyledons (2.5 g) were sliced with a razor blade in three volumes of ice-cold 150 mM Tricine-KOH, pH 7.5, containing 1 mM EDTA and 0.5 M sucrose. The suspension was filtered through three layers of cheese cloth. An aliquot of the flow through fraction was next layered on the top of 35 ml of a continuous sucrose gradient (from 30% to 60% (w/w) sucrose with 1 mM EDTA, pH 8.0), and then centrifuged in a swinging bucket rotor for 150 min at 24,000 rpm (model 55P-72, SRP28SA rotor; Hitachi). After centrifugation, the sucrose gradient was fractionated every 1.5 ml and the fractions were used for analysis.

Glycation of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Its Degradation by cAARE—The glycation of Rubisco was performed by the method described previously (*9*) with slight modifications. Briefly, purified cucumber Rubisco (10 mg) was incubated in 10 ml of 0.5 M Na-PB, pH 7.5, including 3 g of D-glucose and 0.05% (w/v) NaN_3 at 37°C for 30 d. After incubation, the solution was dialyzed extensively against distilled water and then subjected to analysis. Glycation of Rubisco was confirmed by Western blotting with anti-Rubisco antiserum or anti-AGE monoclonal antibody to monitor the aggregation and production of chemical adducts of Rubisco (*9*).

Degradation of the Rubisco protein by AARE was performed with partially purified cAARE. cAARE (11 nmol/min) was mixed with 1 μg of native or glycated Rubisco in 20 μl of 20 mM Hepes-KOH, pH 7.0. After incubation at 37°C, the reaction was stopped by the addition of an equal volume of SDS-sample buffer and subsequent boiling for 1 min. Then, degradation of Rubisco was analyzed by Western blotting using anti-Rubisco antiserum after SDS-PAGE. Quantification of signals was performed with NIH Image software (ver 1.61) after scanning of the membrane.

RESULTS AND DISCUSSION

Cloning and Expression of rAtAARE—In the genome sequenced from *A. thaliana*, a gene homologous to the mammalian gene for AARE was predicted. Therefore, we

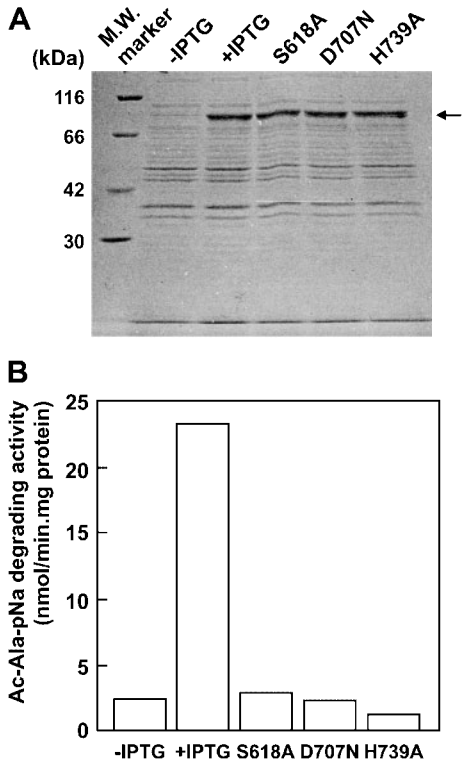

Fig. 2. **Expression of rAtAARE in *E. coli* and measurement of AARE activity.** A: Expression of rAtAARE was performed as described under "MATERIALS AND METHODS." Proteins in crude extracts prepared from *E. coli* with the non-induced wild type (lane, –IPTG), induced wild type (lane, +IPTG), and induced mutant AAREs (as indicated at the top of the figure), were separated by SDS-PAGE and stained with CBB R-250. The arrow indicates the induced AARE protein. B: Ac-Ala-pNa degrading activity in a crude extract prepared from each *E. coli*. The symbols are the same as in panel A.

searched for a gene encoding an entire polypeptide with GENSCAN 1.0 (http://genes.mit.edu/GENSCAN.html) using the sequence of ESSA I FCA contig fragment No. 1 (accession no Z97336), in which the predicted AtAARE is included. As a result, a candidate encoding an entire polypeptide was found, and the ORF was amplified by polymerase chain reaction using two primers, an upper primer (5′-ATGGATTCTTCTGGAACTGATTCGGC-3′) and a lower primer (5′-TCACAGCTTGCAGTACTTG-TTGAACC-3′), with cDNA synthesized from mRNA as a template. The amplified gene was inserted into pQE32 and then sequenced. This gene encoding a 764 amino acid protein of 83.9 kDa exhibited about 31.8% identity with that of AARE from rat (Fig. 1). In particular, the proposed active residues (Ser, Asp, and His) of AARE, called the "catalytic triad residues", were completely conserved. This gene contained a putative signal sequence for microbodies, nuclei or mitochondria, as predicted with the PSORT program (*14*).

In order to obtain evidence that the isolated cDNA encodes a functional AARE, the enzymatic properties of the gene product were determined. *E.coli* strain M15 was transformed with pQE32 containing the AtAARE gene. After incubation in the presence of IPTG for 3h at 25°C, a band corresponding to an apparent molecular mass of 85 kDa, which was consistent with the mass expected from the DNA sequence, was induced (Fig. 2A). This induced protein was confirmed to be AtAARE by amino acid sequencing (data not shown). On enzyme assaying, measurable AARE activity was found in the soluble fraction (Fig. 2B). We attempted to purify the rAtAARE by Ni-Sepharose chromatography, however, non-denatured rAtAARE did not bind to the gel. Moreover, rAtAARE was very liable and thus we could not purify rAtAARE by conventional chromatography. Therefore, we used partially purified rAtAARE, following ammonium sulfate precipitation and DEAE-Toyopearl chromatography, for the analysis.

To confirm that the plant AARE has identical catalytic residues with AAREs of other organisms, the deduced catalytic residues were point-mutated, and the activities of the mutated AARE were measured. As a result, all the mutants exhibited no Ac-Ala-pNa hydrolyzing activity in spite of prominent production of AARE proteins (Fig. 2). These results suggest that AtAARE has three critical amino acid residues identical to those of AAREs of other organisms, and belongs to the prolyl oligopeptidase-related serine-type peptidase family (*15*).

Purification of the Native forms of AAREs from Arabidopsis and Cucumber Plants—Next, to examine the biochemical properties of native plant AAREs, we purified AtAARE and cAARE from plant materials. AtAARE was extracted from shoots of *A. thaliana,* and purified by successive chromatographies on DEAE-Toyopearl, Phenyl-Sepharose, and hydroxyapatite. Since highly purified AtAARE was extremely labile, we could not purify AtAARE homogeneously. cAARE was extracted from young cucumber leaves because cAARE activity was high in such leaves, and was purified by successive chromatographies on DEAE-Toyopearl, Phenyl-Sepharose, hydroxyapatite, and Toyopearl HW-55F gel filtration columns (Table 1). The native molecular masses of the two AAREs

Table 1. **Summary of purification of cAARE.**

	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min.mg)	Purification (-fold)	Yield (%)
Crude extract	13,200	1,556	8.46	1.0	100
Ammonium sulfate (45–60%)	5,840	241	24.2	2.86	44.4
DEAE-Toyopearl	4,720	32.3	146	17.3	35.9
Phenyl-Sepharose	812	5.96	136	16.1	6.2
Hydroxyapatite	795	1.50	530	62.6	6.0
HW-55F[a]	21.0	0.011[b]	1,900	226	0.16

[a]A one-tenth volume of the active fraction from thehydroxyapatite step was loaded on a HW-55F column. [b] Protein was estimated by measurement of the absorbance at 280 nm, with the $E^{1\%}_{1cm}$ value of 10.0.Table 3.

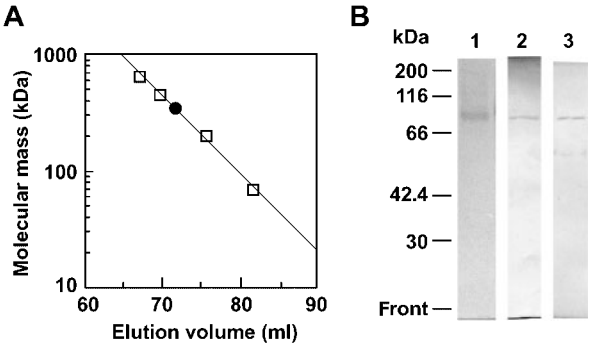

Fig. 3. **Estimation of the molecular masses of AtAARE and cAARE.** A: The molecular masses of native AtAARE and cAARE were estimated on a gel filtration column (HW-55F). The two AAREs showed the same results (closed circle). The molecular weight marker proteins (open squares) were thyroglobulin (660 kDa), apoferritin (440 kDa), amylase (200 kDa), and bovine serum albumin (67 kDa). B: Partially purified AtAARE (2 μg, lane 1) and purified cAARE (0.5 μg, lanes 2 and 3) were subjected to SDS-PAGE on a 10% (w/v) acrylamide gel and stained with CBB R-250 (lane 2) or detected by Western blotting with anti-rAtAARE antiserum (lanes 1 and 3).

were estimated to be about 350 kDa by gel filtration (Fig. 3A). On SDS-PAGE, the molecular mass was estimated to be 82 kDa (Fig. 3B). Therefore, this enzyme is a tetrameric protein composed of identical sized subunits of 82 kDa. Both the AtAARE candidate and cAARE cross-reacted with anti-rAtAARE antibodies, thus AtAARE and cAARE are immunochemically homologous (Fig. 3B). As shown in Table 2, the molecular masses and subunit compositions of plant AAREs are very similar to those of AARE from cow (*16*, *17*), rat (*18*), and man (*19*). The pI of cAARE was determined to be 5.5 by isoelectric focusing (data not shown).

Enzymological Properties of Plant AAREs—AtAARE and cAARE showed similar enzymological properties. The pH optima of the two AAREs were both approximately pH 7.0, and cAARE showed over the half-maximal activity between pH 6.6 and 7.6 (data not shown). This pH optimum is similar to those of other AAREs from mammals and microorganism showing pH optima of 6.9–8.2 (Table 2). The optimum temperatures of the two AAREs were both 37°C (data not shown). The Km values for the cleavage of Ac-Ala-pNa were 1.5 mM (AtAARE) and 4.6 mM (cAARE) at pH 7.0, these values being similar to those for Ac-Ala-pNa of other AAREs (*18*, *19*). Both AAREs were completely inactivated by 1 mM diisopropyl fluorophosphate (DFP). Phenylmethylsulfonyl fluoride was less effective, *i.e.* only half of the activity being inhibited by it at 1 mM. SH-modifying reagents such as *p*-chloromercuribenzoic acid (PCMB, 0.1 mM) and *N*-ethylmaleimide (NEM, 1 mM) also inhibited both AAREs, suggesting that they have a cysteine residue critical for activity. These effects of inhibitors were similar to in the cases of mammalian AAREs (Table 2). cAARE was completely inhibited by 1 mM Zn^{2+} and Cu^{2+}.

Table 3. **Substrate specificity of AAREs for *N*-acylated oligopeptides.**

Substrate (10 mM)	rAtAARE	AtAARE	cAARE
Ac-Ala-Ala	100[a]	100[a]	100[a]
Ac-Ala-Ala-Ala	75.4	81.0	147
Ac-Ala-Ala-Ala-Ala	22.2	126	85.6
Butyryl-Ala-AlaAla	21.2	77	33.6
Formyl-Ala-Ala-Ala	81.3	106	62.4
Ac-Ala-Met	nd[b]	nd	70.4
Ac-Ala	0	0	0
Ac-Met-Ala	292.8	45.0	4.0
Ac-Met-Glu	7.4	6.0	4.3
Ac-Met-Asn	nd	nd	34.1
Ac-Met-Phe	225.8	5.0	0
Ac-Met	0	0	0
Ac-Gly-Gly	0	4.0	37.0
Ac-Gly-Leu	0	0	30.3
Formyl-Gly-Val	nd	nd	30.3

[a]Activity against Ac-Ala-Ala was defined as 100%. [b]Not determined.

Substrate Specificities of AAREs—AtAARE and cAARE efficiently hydrolyzed acyloligopeptides containing N^{α}-acylated terminal residues *in vitro* as well as AAREs from mammals and microorganisms (Table 3). The substrate preference of cAARE was Ac-Ala>Ac-Gly>Ac-Met, and that of AtAARE was Ac-Ala>Ac-Met>Ac-Gly, like the bovine (*16*), rabbit (*20*), and *Rhodotorula* (*22*) ones. Both AAREs also effectively cleaved butyrylated and formylated peptides. On end product analysis by HPLC, we confirmed that cAARE only released an N^{α}-acetylated alanine from the N-terminal end of acetylated oligopep-

Table 2. **Summary of biochemical properties of plant AARE and other AAREs.**

	Plants	Mammals[a]	Microorganisms[b]
Molecular mass	350 kDa (82 kDa × 4 subunits)	300–380 kDa (Bovine, Rat, Human, 75–85 kDa × 4 subunits) 230–245 kDa (Rabbit, 76–80 kDa × 3 subunits)	260 kDa (*Pyrococcus*, 72.4 kDa × 3 subimits)
Optimum pH	7.0	6.9–8.2	7.5 (*Pyrococcus*)
K_m (Ac-Ala-pNA)	1.5 mM (*A. thaliana*) 4.6 mM (Cucumber)	1–9 mM (Rat) 2.13 mM (Human)	20.2 mM (*Pyrococcus*)
Effective inhibitors	DFP, PCMB, NEM, Zn^{2+}, Cu^{2+}	DFP, PCMB, NEM, Hg^{2+}, Cu^{2+}	—
Substrate specificity	Ac-Ala>Ac-Met>Ac-Gly (*A. thaliana*) Ac-Ala>Ac-Gly>Ac-Met (Cucumber)	Ac-Met, Ac-Ala>Ac-Gly (Rat) Ac-Ala>Ac-Met>Ac-Gly (Bovine, Rabbit)	formyl-Met, formyl-Ala>Ac-Met, Ac-Ala (*Pyrococcus*) Ac-Ala>Ac-Met>Ac-Gly (*Rhodotorula*)

[a]Summarized from Refs. *1* and *16–20*. [b]Data from Refs. *21* and *22*.

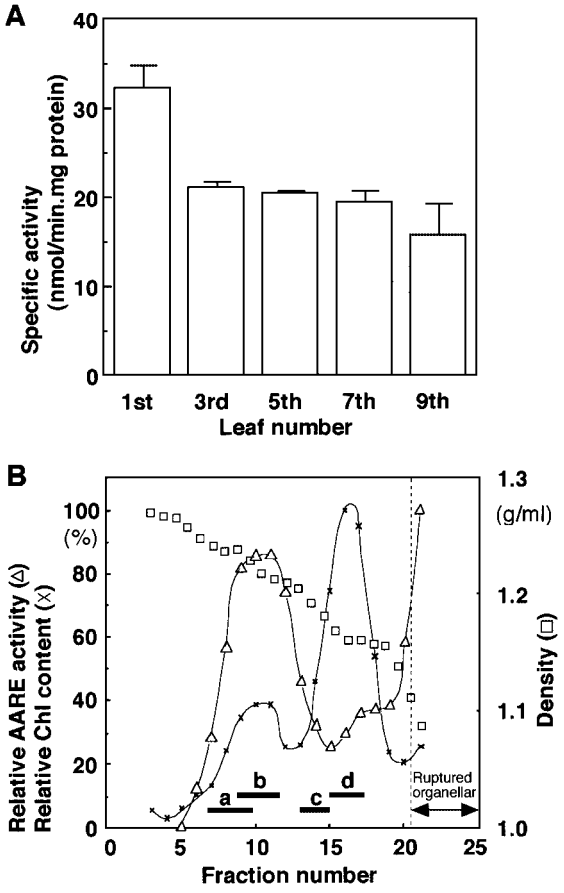

Fig. 4. **Distribution of AARE activity in cucumber plants.** A: AARE activity at each leaf position of an intact plant just after the ninth leaf had emerged. The leaves are numbered from top to bottom. B: A crude organelle suspension prepared from cucumber cotyledons (2.5 g) was separated by sucrose density gradient [30% to 60% (w/w), total 35 ml] centrifugation. The sucrose gradient was fractionated every 1.5 ml from the bottom to top of the tube, the fractions being numbered sequentially. Symbols: AARE activity (triangles), chlorophyll (Chl, ×), and density (squares). Bars indicate fractions containing an organelle marker enzyme or pigment: catalase (a, peroxisomes), Chl and Rubisco (b, intact chloroplasts), cytochrome *c* (c, mitochondria), and Chl but not Rubisco (d, thylakoid of chloroplasts).

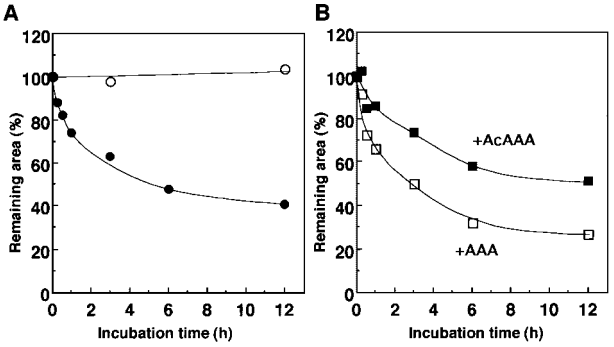

Fig. 5. **Degradation of glycated Rubisco by cAARE.** A: cAARE was incubated with glycated Rubisco (closed circles) or native Rubisco (open circles) for the indicated times. After separation by SDS-PAGE and subsequent Western blotting with anti-Rubisco antiserum, the remaining substrate bands were quantified by densitometry and expressed as "remaining area." B: cAARE and glycated Rubisco were incubated in the presence of 10 mM Ac-Ala-Ala-Ala (closed squares) or Ala-Ala-Ala (open squares). Calculation of the remaining area was performed by the same method as in panel A.

tides (Ac-Ala-Ala-Ala and Ac-Ala-Ala-Ala-Ala), whereas it did not hydrolyze non-acylated peptides (Ala-Ala-Ala, Ala-Ala-Ala-Ala, and data not shown). Thus, a plausible physiological role of AARE is involvement in the final step of degradation of N^{α}-acylated proteins. The slight difference in substrate specificity between rAtAARE and native AtAARE might be reflected by the difference in their conformational structures, that is, the unusual structure of rAtAARE including an additional N-terminal His-tagged peptide, signal peptide and/or mis-folding due to the procaryotic translation machinery in bacteria might lead to different substrate recognition from that by native AtAARE.

Distribution of AARE Activity in Plants—The distribution of AARE activity in plants was examined using cucumber plants because cucumber leaves contain higher AARE activity compared to *A. thaliana,* and they are useful for determining the leaf order and for separating intracellular organelles. AARE activity was detected in all leaves tested, have being a tendency for higher activity in young leaves (Fig. 4A). This result suggests that metabolically active tissues contain high AARE activity.

On subcellular fractionation analysis, distinguishable AARE activity was detected in an oxidative organelle fraction, the stroma of chloroplasts (Fig. 4B). AARE activity was localized to major sites of active oxygen species formation in plants.

Degradation of Glycated Rubisco by Native AARE—Recently, an oxidized protein hydrolase was discovered in the human erythrocyte cytosol (*5*), which was found to be identical to human AARE on comparison of their amino acid sequences and enzymatic activities (*6*). The oxidized protein hydrolase preferentially degrades glycated and oxidatively damaged proteins (*5*). In this study, we examined whether or not cAARE also preferentially degrades glycated proteins using Rubisco as a model stroma protein because cAARE is localized in chloroplast stroma (Fig. 4B).

Glycation of Rubisco was monitored as the aggregation of Rubisco subunits and the emergence of signals reacting with anti-AGE monoclonal antibody (*9*). After 30 d incubation with D-glucose, glycation of Rubisco was evident (data not shown). Therefore, we used 30 days-glycated Rubisco as the substrate for the following experiments. As shown in Fig. 5A, cAARE degraded glycated Rubisco but not native Rubisco. The degradation was completely inhibited by the addition of 1 mM diisopropyl fluorophosphate (data not shown), and, furthermore, the addition of a competitive substrate (10 mM Ac-Ala-Ala-Ala) to the reaction mixture suppressed the degradation of glycated Rubisco, whereas that of a non-substrate, 10 mM Ala-Ala-Ala, did not (Fig. 5B). These results indicated that the degradation of glycated Rubisco was contributed to by cAARE.

Subcellular fractionation analysis and signal sequence prediction indicated that plant AAREs are localized in various organelles in which proteins are likely to undergo chemical modification such as glycation caused by oxida-

tive conditions. Therefore, the physiological roles of plant AAREs might be not only catalysis of the N-terminal hydrolysis of N^{α}-acylpeptides but also the elimination of glycated proteins. In plant chloroplasts, especially under oxidative conditions, glycation possibly occurs through ascorbic acid because ascorbic acid, a potent glycation inducer, is present at high concentrations, *i.e.* 12–25 mM (*23*). Actually, Rubisco was easily glycated under physiological conditions, and glycation led a loss of Rubisco activity and disturbed its native conformation (*9*). AARE might play a role in homeostasis through the degradation of glycated proteins generated in chloroplast stroma.

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