博士学位論文

Molecular Characterization of Ascorbate Peroxidase in Photosynthetic Organisms

(光合成生物におけるアスコルビン酸ペルオキシダーゼの分子特性の解明)

平成8年2月

近畿大学大学院

農学研究科農芸化学専攻

石川孝博

MOLECULAR CHARACTERIZATION OF ASCORBATE PEROXIDASE IN PHOTOSYNTHETIC ORGANISMS

TAKAHIRO ISHIKAWA

1996

I wish to express my sincere gratitude to Dr. Shigeru Shigeoka, Professor of Faculty of Agriculture, Kinki University, for his kind guidance, valuable advice, stimulating discussion and critical review throughout the work including the manuscript of this thesis.

I wish to thank Dr. Toshio Mitsunaga, Professor of Faculty of Agriculture, Kinki University, for his kind advice and encouragement.

Thanks are due to Dr. Osamu Hirayama and Dr. Akio Enoki, Professors of Faculty of Agriculture, Kinki University, for reading the entire text in its original form.

I am thankful to Dr. Yoshihisa Nakano, Professor of Applied Biological Chemistry, University of Osaka prefecture, and Dr. Akiho Yokota, head researcher of the Research Institute of Innovative Technology for the Earth (RITE), for their kind suggestions and valuable discussions throuout the work.

I greatly thank to Dr. Kozi Asada, the Reserach Institute for Food Science, Kyoto University, for generously providing the guaiacol peroxidase from spinach leaves.

I am greatly indebted to Dr. Hiroaki Kohno, Research Laboratories, Kyowa Medex Co., Ltd., for his kindly guidance and technical support of the production of monoclonal antibody.

Finally, special thanks are due to Toru Takeda and Kosuke Sakai, for their many helpful collaborations and valuable advice. Thanks are also due to all the past and present members of the laboratory of the Nutrition Chemistry in the Faculty of Agriculture, Kinki University, for their kind cooperation and help.

ABBREVIATIONS

AsA	reduced ascorbate
AsAP	ascorbate peroxidase
cAsAP	cytosolic ascorbate peroxidase
sAsAP	stromal ascorbate peroxidase
tAsAP	thylakoid-bound ascorbate peroxidase
BSA	bovine serum albumin
CHI	cycloheximide
CCP	cytochrome *c* peroxidase
DAsA	dehydroascorbate
ELISA	enzyme-linked immunosorbent assay
FBPase	fructose 1,6-bisphosphatase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GP	guaiacol peroxidase
GSH	reduced glutathione
GSHP	glutathione peroxidase
H_2O_2	hydrogen peroxide
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-ethanesulfonic acid
HRP	horseradish peroxidase
HU	hydroxyurea
IPTG	isopropyl-β-D-thiogalactopyranoside
mAb	monoclonal antibody
MES	2-(*N*-Morpholino)ethanesulfonic acid
MDAsA	monodehydroascorbate
$^1O_2$	singlet oxygen
O_2^-	superoxide
OH•	hydroxyl radicals
PAGE	polyacrylamede gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PVDF	polyvinylidene difluoride
RACE	rapid amplification of cDNA ends
Ru5P-K	ribulose-5-phosphate kinase
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBS	Tris-buffered saline
Tris	tris(hydroxymethyl)methylglycine

CONTENTS

CHAPTER I

Introduction

Generation of active oxygen species

Most of living organisms require dioxygen for the efficient production of energy. Free dioxygen appeared in the Earth's atmosphere in significant amounts about 2×10^9 years ago, probably due to the evolution of dioxygen-evolving photosynthetic organisms. As the dioxygen level of the atmosphere rose, it also expected living matter to oxygen toxicity. Active oxygen species, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•), inactivated enzymes and damage important cellular components (Halliwell and Gutterridge 1985). Although the formation of active oxygen species is generally considered to be detrimental to cellular function, these moleculars are formed in normal cell metabolism and their production is a regulated cellular phenomenon (Fig. 1.1). Plant cells are especially prone to oxygen-toxicity effects, because dioxygen concentration into their chloroplasts in the light will always be greater than that in the surrounding atmosphere, due to dioxygen production in photosystem II. Moreover, the electron-transport chain of chloroplasts, like that of mitochondria and endoplasmic reticulum, can be achieved by the reduction of dioxygen at the acceptor side of photosystem I in the Mehler reaction (Mehler 1951). This forms the potentially deleterious O_2^- radicals. Therefore, photosynthetic organisms have evolved protective mechanisms against oxidative stress caused by active oxygen species.

Protection against active oxygen species

To counteract the toxicity of active oxygen species, a highly efficient antioxidative defense system, composed of both non-enzymic and enzymic constituents, is present in all photosynthetic organisms. The non-enzymic antioxidants are generally small molecules. Ascorbate (AsA) plays a pivotal role in the destruction of active oxygen species, particularly H_2O_2. In addition, the tripeptide glutathione (GSH) and lipophilic antioxidant α-tocopherol together with the carotenoid pigments fulfill essential antioxidant functions. The enzymic antioxidative components are a prerequisite for life in dioxygen ; they include superoxide dismutase (SOD), catalase, ascorbate peroxidase (AsAP), electron donor-nonspecific peroxidases like guaiacol peroxidase (GP), and the enzymes involved in the synthesis and regeneration of the reduced forms of antioxidants. The protective action of catalase is limited because of 1) its discrete localization in the peroxisomes, 2) its relatively poor affinity for its substrate and 3) its sensitivity to light induced inactivation. Glutathione peroxidase (GSHP), an important enzyme in the H_2O_2-detoxification system of animals located in the cytosol and mitochondria, is largely

absent from plant tissues. However, GSHP has been found for the first time in the green alga *Chlamydomonas reinhardtii* (Yokota et al. 1988, Shigeoka et al. 1991b).

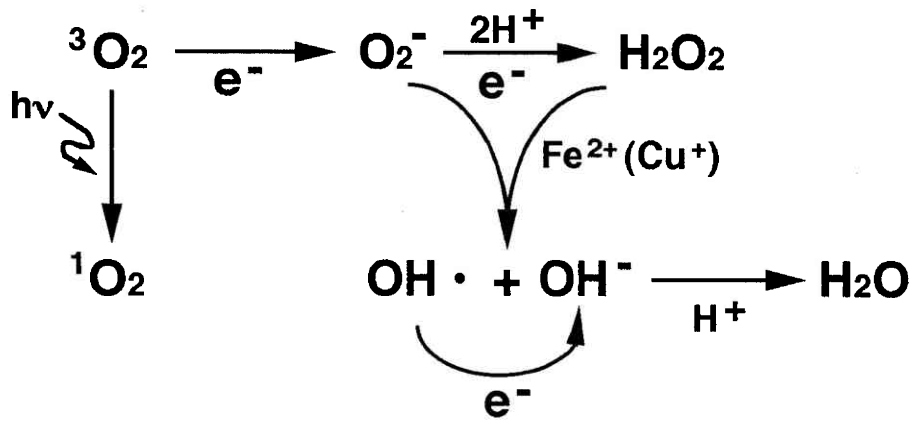

Fig. 1.1 The formation of active oxygen species in the reduction of O_2 to H_2O_2.

Furthermore, the isolation of cDNAs with high homology to GSHs in *Nicotiana sylvestris* (Criqui et al. 1992) and *Citrus* (Holland et al. 1993) suggests that a functional GSH cycle also may exist in plants. The native activities and subcellular localization of these GSHPs in plants have not still to be established. Oxidative damage to the chloroplasts and other cellular compartments by H_2O_2 is minimized by the AsA-GSH cycle including AsAP (Fig. 1.2). In this cycle, AsAP reduces H_2O_2 to form water and monodehydroascorbate (MDAsA). MDAsA spontaneously disproportionates to AsA and dehydroascorbate (DAsA; $10^5\ M^{-1}\ s^{-1}$ at pH 7.0). MDAsA is also directly reduced to AsA by the action of NAD(P)H-dependent MDAsA reductase (EC 1.6.5.4). DAsA reductase (EC 1.8.5.1) utilizes GSH to reduce the DAsA and thereby regenerate the AsA. The GSH is then regenerated by GSH reductase, utilizing reducing equivalents from NAD(P)H.

Ascorbate peroxidase

The enzyme AsAP (E.C 1.11.1.11) has been found in higher plants such as spinach (Nakano and Asada 1987, Tanaka et al. 1991), pea (Gerbling et al. 1984, Mittler and Zilinskas 1991a), maize (Koshiba 1993), tea (Chen and Asada 1989) and legumes root nodule (Dalton et al. 1987) and also detected in eukaryotic algae including *Euglena* (Shigeoka et al. 1980a) and *Chlamydomonas* (Yokota et al. 1988, Shigeoka et al. 1991b) and certain cyanobacteria (Tel-Or et al. 1986, Miyake et al. 1991). In addition to photosynthetic organisms, AsAP has been found in the protozoan *Trypanosoma cruzi* (Boveris et al. 1980), but not in fungi and mammals.

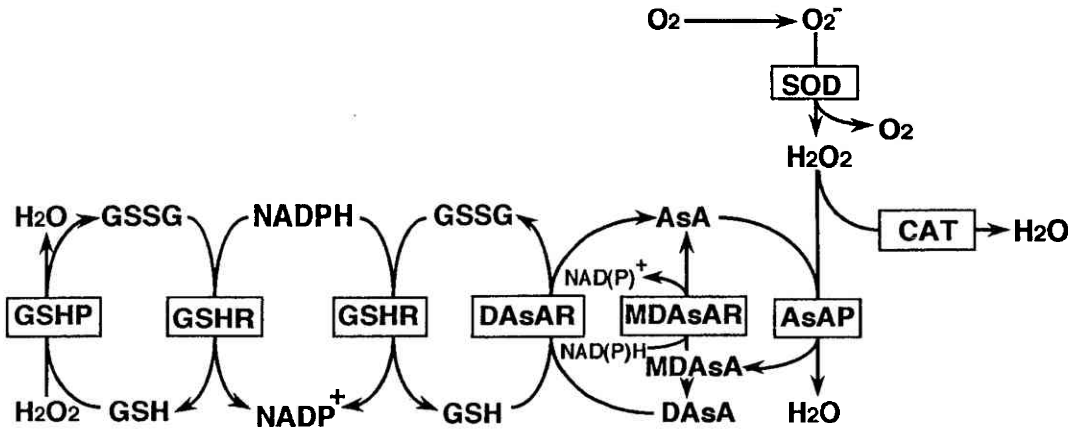

Fig. 1.2 Ascorbate-glutathione cycle. Abbreviations used following: AsAP, ascorbate peroxidase; MDAsAR, monodehydroascorbate reductase; DAsAR, dehydroascorbate reductase; GSHR, glutathione reductase; GSHP, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase

AsAP catalyzes the oxidation of AsA by H_2O_2, generating two molecules of the MDAsA and has major role in preventing the accumulation of toxic levels (μM) of H_2O_2 in plant cells.

$$2AsA + H_2O_2 \longrightarrow 2MDAsA + 2H_2O$$

AsAP has been known as two type of isozymes, that is, chloroplastic and cytosolic forms (Asada 1992). In chloroplasts of higher plants, which lack catalase, AsAP occurs in the stroma in a soluble form and also in the thylakoids in a membrane-bound form (Miyake et al. 1993). AsAP is a hemeprotein just as the GP, but it is clearly distinct from the GP in terms of enzymlogical and molecular properties (Foyer et al. 1991, Asada 1992).

In this study

Unfavorable environmental conditions such as high light intensities, drought stress, air pollutants, low temperatures, etc., can result in an increase of oxidative stress to photosynthetic organisms. Although much of information concerning AsA-GSH cycle has focused on its role in the prevention of oxidative damage in the photosynthetic organisms (Hérouart et al. 1993, Foyer et al. 1994a and 1994b, Allen 1995), and several studies have demonstrated an increase in AsAP activity in response to environmental stresses (Tanaka et al. 1985, Schöner and Krause 1990, Cakmak 1994), little is known about the molecular mechanism and regulation of AsAP isozymes underlying its response to these stresses.

Using a higher plant as an investigating system was found to be difficult due to the structural complexity of higher plants, the many factors affecting growth anf the length of the life cycle. Moreover, in higher plants, the situation is ofen considerably complicated by the presence of a large number of isoenzyme forms encoded by different genes. At the point of these views, in *Euglena gracilis*, which lacks catalase, the antioxidant enzymes involved in the AsA-GSH cycle occur only in the cytosol but not in the chloroplasts (Shigeoka et al. 1980b, 1987c). These findings indicate that *Euglena* cells may be a useful species for elucidating in detail the physiological role of cytosolic AsAP in photosynthetic organisms. While a number of cDNAs for cytosolic AsAP have been isolated and characterized from plants (Mittler and Zilinskas 1991, Kubo et al. 1992, Webb and Allen 1995), no cDNAs that encode chloroplastic AsAP have yet been identified. For this reason, little is known about the molecular mechanism underlying its response to environmental stresses.

In the present thesis, I studied the followings to provide important informations on molecular characterization and regulation mechanism of AsAP in *Euglena gracilis* and higher plants;

1) Molecular properties of *Euglena* AsAP
2) Production of monoclonal antibodies (mAbs) against *Euglena* AsAP
3) Effect of iron and light on the induction of AsAP
4) cDNA cloning encoding AsAP isozymes from spinach using *Euglena* AsAP mAbs as a probe

CHAPTER II

Molecular Characterization of *Euglena* Ascorbate Peroxidase Using Monoclonal Antibody

Ascorbate peroxidase (AsAP) has been known as two types of isozymes, that is, chloroplastic and cytosolic forms (Asada 1994). In chloroplasts, which lack catalase, AsAP occurs in the stroma in a soluble form and also in the thylakoids in a membrane-bound form and is the effective system for removal of H_2O_2 generated by photosynthetic processes (Asada 1994). In contrast, the detailed physiological roles of cytosolic AsAP remain unclear. Shigeoka et al. (1980b, 1987c) have reported that in *Euglena*, which lacks catalase, the antioxidant enzymes involved in the AsA-GSH cycle occur only in the cytosol but not in the chloroplasts. It has been reported that part of the decomposition of H_2O_2 can be accounted for by the peroxidase-catalyzed reaction linked to the photosynthetic electron transport of *Euglena* chloroplasts (Miyake et al. 1991). These findings indicate that *Euglena* cells may be a useful species for elucidating in detail the physiological role of cytosolic AsAP in photosynthetic organisms. Previously, Shigeoka et al. (1980a) have been reported the partial purification and some enzymological properties of AsAP from *Euglena gracilis*. In this chapter, I report the purification of AsAP from *E. gracilis*, the production and characterization of monoclonal antibodies raised against the *Euglena* AsAP and its partial primary amino acid sequence.

Materials and Methods

Materials —— The materials used were obtained from the sources indicated: BALB/c mice (CLEA, Osaka, Japan), peroxidase-conjugated goat anti-mouse Igs (Organon Teknika Corp., West Chester, PA), and ascorbic acid (Wako Chemical, Osaka, Japan). All other chemicals were of analytical grade and obtained from commercial sources. Plant materials were purchased from a local market.

Cell culture —— *Euglena gracilis*, strain Z, maintained at 26°C under illumination (35 μE m^{-2} s^{-1}), was cultured in Koren-Hutner medium at 26°C for 6 d, by which time the stationary phase was reached (Shigeoka and Nakano 1993).

Enzyme assay —— AsAP activity was assayed at 32°C in 2 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 6.3), 0.4 mM AsA, 0.1 mM H_2O_2 and the enzyme (Shigeoka et al. 1980a). The reaction was initiated by the addition of the enzyme. The oxidation of AsA was followed by a decrease in the absorbance at 285 nm (5.8 mM^{-1} cm^{-1}). The electron donor specificity of *Euglena* AsAP was examined according to Shigeoka et al. (1980a). The activities with organic peroxides were assayed

in the same reaction mixture as that of H_2O_2, but H_2O_2 was replaced by 0.1 mM *t*-butyl hydroperoxide or 0.1 mM cumene hydroperoxide. Hydroxyurea at a final concentration of 80 mM was used as a suicide inhibitor in the same mixture (Chen and Asada 1990).

Enzyme purification—— All purification procedures were performed at 4°C. *Euglena* cells (40 g wet/wt) were harvested by centrifugation, resuspended in 100 ml of 50 mM potassium phosphate buffer (pH 6.3) containing 20% sucrose (w/v), 1 mM EDTA and 1 mM AsA (buffer A), and sonicated (10 kHz) for a total of 15 min with 2 intervals of 5 min each. This lysate was centrifuged at 15000 × g for 20 min to remove cell debris. The supernatant was subjected to ultracentrifugation at 100000 × g for 30 min. The obtained supernatant was loaded onto a DEAE-cellulose column (2.5 × 45 cm) equilibrated with buffer A. The column was eluted with 300 ml of a linear gradient of 0-300 mM KCl. Active fractions were collected and subjected to $(NH_4)_2SO_4$ precipitation at 30% saturation. The precipitate was removed by centrifugation and the supernatant was loaded onto a butyl-Toyopearl column (2 × 16 cm) equilibrated with 20% saturated $(NH_4)_2SO_4$ in buffer A. The enzyme activity was eluted with a descending gradient of $(NH_4)_2SO_4$ from 20 to 0% saturation in 300 ml of buffer A. The active fraction was pooled and dialyzed against 20 mM potassium phosphate buffer (pH 6.3) containing 1 mM EDTA and 1 mM AsA for 4 h with two changes of buffer A. The dialyzed fraction was loaded onto a column (2 × 16 cm) of DEAE-Sephacel equilibrated with 20 mM MES buffer (pH 6.3) containing 10% sucrose, 1 mM EDTA and 1 mM AsA (buffer B). The AsAP activity was eluted from the column with a 300 ml linear gradient of 0 to 0.3 M KCl in buffer B. Active fractions were combined and loaded onto a column (1 × 10 cm) of hydroxylapatite equilibrated with buffer B. The AsAP activity was eluted from the column with a linear gradient of 0 to 20 mM phosphate in buffer B. The active fraction was pooled and concentrated by ultrafiltration using a Centricon-10 concentrater (Amicon). The preparations were applied to a gel filtration column (2.6 × 100 cm) of Sephacryl S-300, equilibrated with 20 mM potassium phosphate buffer (pH 6.3) containing 10% sucrose, 1 mM EDTA, 1 mM AsA and 0.1 M NaCl, and eluted with the same buffer. The purified enzyme was stored at -20°C prior to use.

Protein assay—— Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Digestion of AsAP and peptide separation—— Peptides of the purified AsAP were generated by digesting 500 pmol of protein with *Achromobacter* lysyl endopeptidase (Wako Chemicals, Osaka, Japan) at an enzyme:substrate ratio of 1:100 for 20 h at 37°C in 0.1 M Tris-HCl buffer (pH 8.9). The resulting peptide mixtures were separated by a reverse-phase HPLC with a column of μ Bondasphere 5μ C18 300A (3.9 × 150 mm, Millipore). Gradient elution was done at 0.5 ml/min with 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 60% acetonitrile.

Protein sequencing—— Primary sequence analysis was performed by automated Edoman degradation with a model 477A sequencer from Applied Biosystems using the manufacturer's standard programming and chemicals (Shigeoka and Nakano 1991a).

Preparation of monoclonal antibodies—— Male BALB/c mice (6 weeks old) were immunized three times with the purified AsAP (approximately 200 μg). Spleen cells from the immunized mice were fused with P3/U1 murine myeloma cells. The fused cells were incubated with HAT medium (GIT medium supplemented with 0.1 m M hypoxantine, 0.4 μM amethopterin, and 16 μM thymidine). Culture supernatants of the hybridoma were screened using an ELISA and immunoblotting. Positive hybridoma cells were further selected on the basis of specificity and were cloned by limiting dilution. After repeated screening, eight clones were obtained. Stable hybridoma clones were propagated as ascites tumors in BALB/c mice. The isotype of the monoclonal antibodies was determined on an ELISA using a Mouse Typer Kit (Bio-Rad).

ELISA—— In the standard assay, 100 μl of Tris-buffered saline (TBS) containing 1 μg of antigen protein was placed in each well of a 96-well microtiter plate and incubated 18 h at 4°C. After removing the antigen fluid, the wells were blocked with TBS containing 2% BSA for 2 h at room temperature. After washing the wells three times with TBS (pH 7.8) containing 0.05% Tween-20, the monoclonal antibody was reacted for 2 h at room temperature, followed by peroxidase-conjugate goat anti-mouse Igs antibody for 2 h at room temprature. Finally, the remaining peroxidase activity was determined using 5-amino-salicylic acid as substrate. The results were monitored spectrophotometrically as optical density on an ELISA plate reader (Bio-Rad, model 2550) with a filter at 410 nm.

Immunoblotting—— Proteins were separated using SDS-PAGE (10% gels) with 2-mercaptoethanol according to Leammli (1970) and blotted onto an Immobilon-P transfer membrane (PVDF, pore size 0.45 μm, No. IPVH 304FO, Millipore, Bedford, MA) using a semidry electroblotting system according to the manufacturer's instructions (Bio-Rad). Immunoblotting was performed according to the method of Towbin et al. (1979). Antibody against mouse Igs, conjugated with peroxidase, was used as the second antibody.

Results and Discussion

Purification of ascorbate peroxidase from Euglena——The purification procedures of AsAP from *Euglena* cells are shown in Table 2.1. During purification, the enzyme activity was eluted from all columns as a sharp single peak. This result agreed with the previous study and supported the belief that *Euglena* cells contained only one type of AsAP (Shigeoka et al. 1980a). The enzyme was purified approximately 800-fold over

the crude extract with a yield of 4%. The purification was repeated several times with similar results. The specific activity with AsA and H_2O_2 was 475.6 μmol min^{-1} mg $protein^{-1}$, which was comparable to those of cytosolic enzymes from tea leaves (Chen and Asada 1989), potato (Elia et al. 1992), pea (Mittler and Zilinskas, 1991a), and legumin root noodles (Dalton et al. 1987). The molecular mass of AsAP was approximately 58 kDa by SDS-PAGE and gel filtration on a calibrated Sephacryl S-300 column (Fig. 2.1), indicating that the *Euglena* AsAP exists as a monomeric form in its native state.

Table 2.1. Purification of AsAP from *Euglena gracilis Z*

Step	Total protein (mg)	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg $protein^{-1}$)	Yield (%)
Crude extract	5185.0	2854.8	0.6	100
Ultra-centrifugation	2832.0	2421.9	0.9	85
DEAE-Cellulose	816.0	2411.9	3.0	84
30%$(NH_4)_2SO_4$	552.0	2012.8	3.7	71
Butyl-Toyopearl	92.4	1250.2	13.5	44
DEAE-Sephacel	34.8	603.1	17.3	21
Hydroxylapatite	2.3	335.3	147.7	12
Sephacryl S-300	0.2	111.3	475.6	4

Cytosolic AsAPs purified from tea (Chen and Asada 1989), pea (Mittler and Zilinskas, 1991a), and legume root nodules (Dalton et al. 1987) indicate native molecular mass of 57 kDa, 57.5 kDa, and 47 kDa, respectively. The enzymes from pea and legume root nodules also have a subunit molecular mass of 30 kDa by SDS-PAGE, indicating that general cytosolic AsAPs are dimers. In contrast, the chloroplastic isozymes of tea stromal (Chen and Asada 1989) and spinach thylakoid-bound AsAP (Miyake et al. 1993) have a molecular mass of 34 kDa and 40 kDa, respectively, by SDS-PAGE and gel filtration. In this respect, *Euglena* AsAP seems to be more similar to a chloroplastic type than to a cytosolic type, though the molecular mass of *Euglena* enzyme is twice that of the cytosolic type.

Table 2.2 shows a comparison of some properties of AsAPs purified from *Euglena* and higher plants. AsAP isozymes of higher plants have distinguished specificity for the electron donor for AsA. The chloroplastic isozyme is specific to AsA. In contrast, the cytosolic isozyme of higher plants can oxidize pyrogallol at an appreciable rate (Asada 1994). *Euglena* AsAP was able to catalyze the oxidation of pyrogallol at a 2-fold higher rate than that of AsA. One of the characteristic properties of higher plant AsAP is inactivation in the absence of AsA (Chen and Asada 1989). This is especially the case for chloroplastic AsAP, whose half-inactivation time is only 15s (Asada 1994).

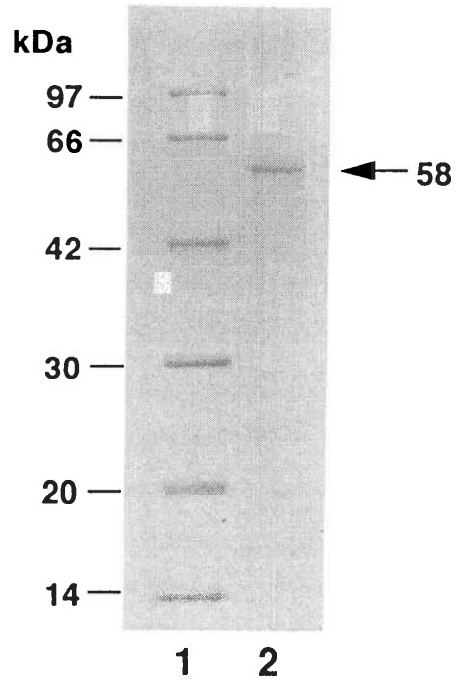

Fig. 2.1. SDS-PAGE analysis of the purified AsAP from *Euglena*. Polyacrylamide gel concentration was 12.5%. Protein standards (lane 1) and 5 μg of purified enzyme (lane 2) were subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250. The arrow indicates the AsAP.

Table 2.2. Comparison of some enzymatic properties of *Euglena* AsAP and thylakoid-bound (t), stromal (s), and cytosolic (c) AsAPs and guaiacol peroxidase (GP) from higher plants. Enzymatic assay was performed as described in "Materials and Methods".

	Euglena	tAsAP Spinach[a]	sAsAP Spinach[b]	cAsAP Pea[c]	GP Spinach[d]
Molecular mass (kDa)	58 (monomer)	40 (monomer)	30 (monomer)	28 (dimer)	34 (monomer)
Donor specificity (%)					
AsA	100	100	100	100	100
Iso-AsA	64.5		93		109
GSH	0	0	0		0
Cyt *c*	0	0	0		0
NAD(P)H	0	0	0		234
Pyrogaroll	200	5.5	0.7	174	46980
Guaiacol	0		3.3	40	9082
Peroxide specificity (%)					
H2O2	100	100	100	100	100
t-BOOH	68.1	n.d.	0	n.d.	n.d.
CumOOH	52.1	n.d.	n.d.	n.d.	n.d.
Inhibition by					
Suicide inhibitors	+	+	+	+	–
Ascorbate depletion	slow	rapid	rapid	slow	none

n.d. : not determined

[a]Miyake et al. 1993, [b]Nakano and Asada 1987, [c]Mittler and Zillinskas 1991, [d]see Chapter VII

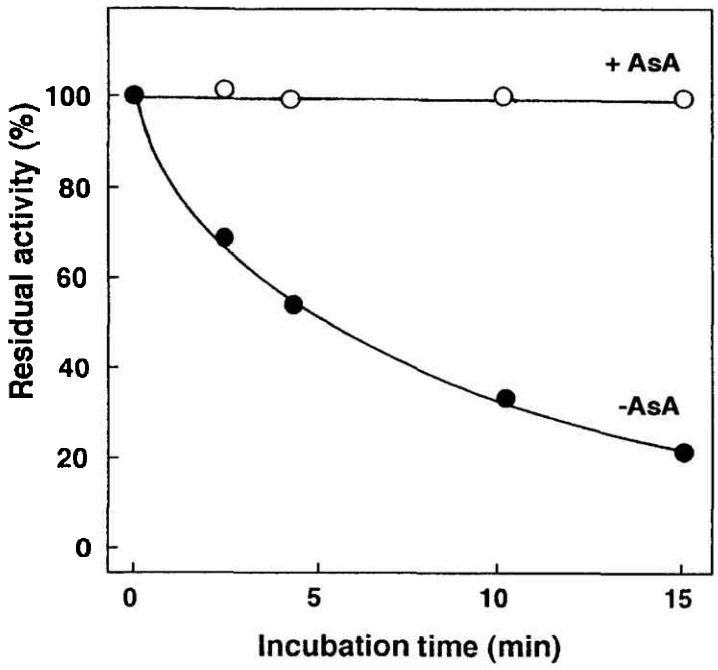

Fig. 2.2. The inactivation of purified AsAP from *Euglena* by deplation of AsA. Purified AsAP stored in a medium containing 20 mM potassium phosphate (pH 6.3), 100 μM AsA and 10% sucrose was diluted with 20 mM potassium phosphate (pH 6.3) at the final AsA concentration of below 10 μM (-AsA). After the dilution, 0.5 mM AsA was added at the indicated times and AsAP activity was assayed according to "Materials and Methods". Where indicated (+AsA), the dilution was done with the buffer containing 0.5 mM AsA.

Cytosolic AsAP is more stable than the chloroplastic type. The half-inactivation time of the purified *Euglena* AsAP was approximately 5 min (Fig. 2.2).

The *Euglena* AsAP also reduced *t*-butyl hydroperoxide and cumene hydroperoxide as an electron acceptor in the presence of AsA, which was in agreement with the result described previously (Shigeoka et al. 1980a). It has been reported that the AsAPs of some cyanobacteria reduce various organic hydroperoxides as well as H_2O_2 (Tel-Or et al. 1986). In Chapter III, *Euglena* cells show an absolute requirement for iron for growth, AsAP activity is not observed in iron-deficient *Euglena* cells, and that lipid peroxides (thiobarbituric acid-reactive substances) in iron-deficient cells are approximately 2.6-fold greater than those in iron-sufficient cells. These results suggest that the *Euglena* AsAP, like glutathione peroxidase in animals and *Chlamydomonas* (Shigeoka et al. 1991b), may serve to protect the cell membrane by reducing the peroxide compounds generated endogenously from unsaturated fatty acids. A similar situation may also occur in eukaryotic algae and cyanobacteria.

Primary amino acid sequence analysis of Euglena *ascorbate peroxidase* —— The N-terminal sequence of the 35 amino acids of *Euglena* AsAP was determind by direct analysis of the undegraded enzyme as follows: ELPTWVPDFVKGLLEPPQPPYT-LAEIDQVPWGQLA. A comparison of the N-terminal sequence to 21 residues of the *Euglena* AsAP with cytosolic and chloroplastic AsAPs from several plant sources indicates no significant sequence similarity (Fig. 2.3). This result may be in part due to the difference in molecular weight between *Euglena* AsAP and higher plant AsAPs, because the molecular mass of *Euglena* AsAP is approximately 18 to 28 kDa higher than those of higher plant AsAPs. Screening of the N-terminal sequence in the Swiss-Prot database revealed that its similarity to classical plant peroxidases, such as guaiacol peroxidase, was also very low.

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                1         5         10        15        20
E.AsAP          E-L-P-T-W-V-P-D-F-V-K-G-L-L-E-P-P-Q-P-P-Y-

P.cAsAP         G-K-S-Y-P-T-V-S-P-D-Y-Q-K-A-I-E-K-A-K-R-K-
S.cAsAP         G-K-S-Y-P-T-V-S-E-N-Y-Q-K-S-I-E-K-A-R-R-K-
A.cAsAP         T-K-N-Y-P-T-V-S-E-D-Y-K-K-A-V-E-K-C-R-R-K-

S.sAsAP         Y-A-S-D-P-A-Q-L-K-N-A-R-E-D-I-K-E-L-L-Q-R-
S.tAsAP         Y-A-S-D-P-A-Q-L-K-N-A-R-E-D-I-K-E-L-L-Q-S-
T.sAsAP         F-A-S-D-P-D-E-L-K-S-A-R-E-D-I-K-E-L-L-N-T-
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Fig. 2.3. The N-terminal amino acid sequences of AsAPs from *Euglena* and higher plants. The sequences of N-terminal amino acids of AsAPs were determined with a model 477A automated gas phase sequencer (Shigeoka and Nakano 1993). Amino acid sequences were aligned to obtain maximal similarity. Shaded area shows homologies. E.AsAP, *Euglena* AsAP (this study); P.cAsAP, Pea cytosolic AsAP (Mittler and Zilinskas 1991); S.cAsAP, Spinach cytosolic AsAP (Chapter VII); A.cAsAP, *Arabidopsis* cytosolic AsAP (Kubo et al. 1992); S.sAsAP, Spinach stromal AsAP (this study); S.tAsAP, Spinach tylakoid-bound AsAP (Miyake et al. 1993); T.sAsAP, Tea stromal AsAP (Chen and Asada 1989).

In order to obtain any internal primary structure information, the *Euglena* AsAP was cleaved at the lysine residues by lysyl endopeptidase. The peptides were resolved by a reverse-phase HPLC using a gradient of 0 to 60% acetonitrile. Approximately 30 peptides were detected at 210 nm and well-resolved peaks (21 peptides) were collected and sequenced (Fig. 2.4, 2.5). Sequences containing a total of 195 residues were determined, which represented 37.5% of the *Euglena* AsAP, as deduced from the molecular mass. The partial amino acid sequences of *Euglena* AsAP were compared with those of AsAPs from plant sources and cytochrome *c* peroxidase (CCP) from yeast, which were deduced previously from their respective cDNAs, and those of chloroplastic AsAP purified from tea leaves (Fig. 2.6).

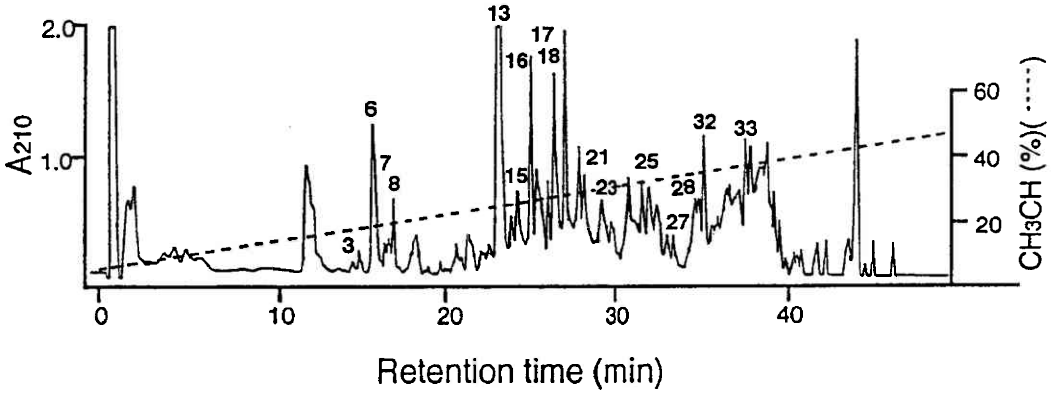

Fig. 2.4. Separation of lysyl endopeptidase digests of *Euglena* AsAP by reverse-phase HPLC. The digested AsAP was applied to a column of μ Bondasphere 5μ C18 300A (3.9 × 150 mm, Millipore) and eluted with a linear gradient of acetonitrile (0-60%, 1 %/min) in 0.1% trifluoroacetic acid.

P- 3	YAGVMAG	P-18b	EAVERGRLPDATQT
P- 6a	GPSFWK	P-21a	XTLGTS
P- 6b	VVATEVSGGPK	P-21b	YSWVSTA
P- 7	TLVTSSAGK	P-23	YFATAT
P- 8	YQQSDTGPVA	P-25	DLNAFFTN
P-13	QVAFFRVY	P-27	VFDNXYFK
P-15	HQKLVPPAEXV	P-28a	DEVAYF
P-16a	WTSVTDK	P-28b	GPFYTMRFGK
P-16b	TSDLALLMD	P-32	VPELPVAFDRV
P-17	MXWVK	P-33	YNTFFVNLLNK
P-18a	ELPTWVPDFVK		

Fig. 2.5. Peptide sequence of *Euglena* AsAP. Peptides are numbered according to the elution profiles by reverse-phase HPLC, and the letters of the alphabet indicate the peptide peak separated by rechromatography, as described in the text. P-18a corresponded with the N-terminus sequence of the undegraded enzyme.

It has been reported that the primary amino acid sequences of chloroplastic and cytosolic AsAPs have considerable homology with that of yeast CCP (Mittler and Zilinskas 1991b, Kubo et al. 1992, Chen et al. 1992). Welinder (1992) has described that AsAP and CCP belong to the same class I peroxidases, which are distinguished from classical plant peroxidases (class III). Six peptides (P-6b, P-16, P-18, P-27, P-28a, and P-28b) derived from *Euglena* AsAP showed a high degree of homology to cytosolic AsAPs from *Arabidopsis* (Kubo et al. 1992) and spinach (Chapter VII). In Chapter VII, I have demonstrated that cDNA encoding a new type of AsAP (SAP1) is isolated from spinach and its recombinant enzyme shows properties similar to those of cytosolic AsAPs. P-6b, P-16, and P-27 exhibited 63.6%, 55.6%, and 62.5% homology with SAP1. Especially, peptide P-18b showed 50.0%, and 42.9% homology with both tea stromal AsAP and yeast CCP, respectively (Chen et al. 1992, Kaupt et al. 1982). Peptide P-6b and P-27

exhibited highly homologous sequences with yeast CCP (45.5%) and tea stromal AsAP (85.7%), respectively. These results indicate that the *Euglena* AsAP exists in highly homologous regions with the AsAPs of higher plants and the CCP of yeast.

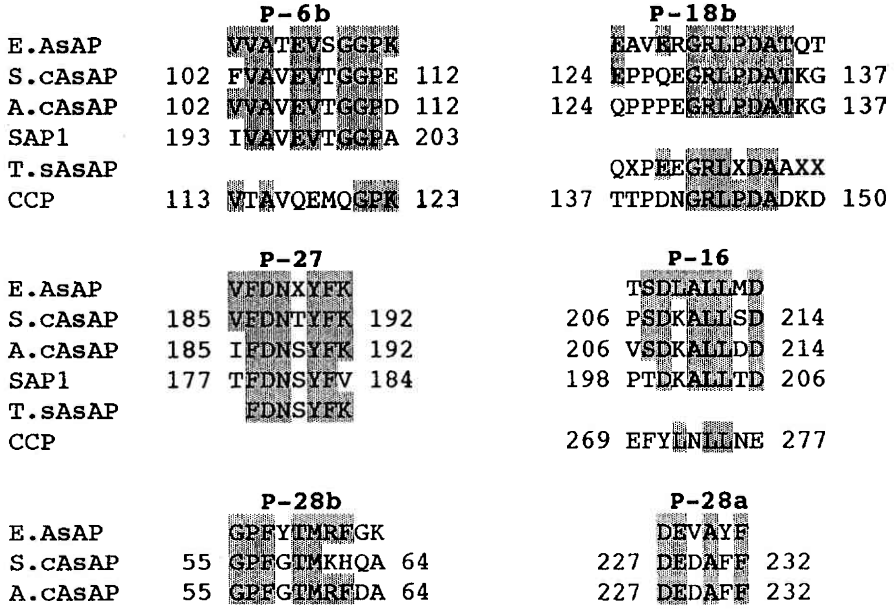

Fig. 2.6. Alignment of amino acid sequence of the peptides from *Euglena* AsAP, higher plant AsAP isozymes, and yeast CCP. E.AsAP, *Euglena* AsAP (this study); SAP1, spinach AsAP (Chapter VII); S.cAsAP, spinach cytosolic AsAP (Chapter VII); A.cAsAP, *Arabidopsis* cytosolic AsAP (Kubo et al. 1992); T.sAsAP, Tea stromal AsAP (Chen and Asada 1989); CCP, yeast CCP (Kaupt et al. 1982). Shaded area shows homologies.

Production of monoclonal antibodies against Euglena *ascorbate peroxidase* —— A purified *Euglena* AsAP was used for the immunization of mice. Hybridoma cultures were screened for the production of antibodies specific to purified AsAP by both ELISA and immunoblots. Eight monoclonal hybridomas (EAP1-8) were chosen for expansion and passage into mice for ascites fluid production. For the designation of these monoclonal antibodies (mAb) see Table 2-3. Subtyping analysis showed that two mAbs (EAP1, and EAP2) were of the IgG1 subclass, whereas the other mAbs (EAP3-8) were of the IgM type. All mAbs had κ light chains. The ascites fluids from these mAbs were titrated by $1/(2^n)$ dilutions under optimal conditions. The EAP1 and EAP2 gave 10^{-3} and 5×10^{-6} dilutions, respectively, to 50% binding in the ELISA (Fig. 2.7). The five IgMs (EAP4-8) responded much more weakly. Incubation of the purified *Euglena* AsAP with two IgGs (EAP1 and EAP2) had little effect on the activity of the enzyme, indicating that these antibodies were not directed toward the active site of AsAP. After SDS-PAGE of crude *Euglena* extracts, followed by immunoblotting, all eight mAbs recognized a 58 kDa band corresponding to the size of the *Euglena* AsAP (Fig. 2.8).

Table 2-3. Characterization of monoclonal antibodies raised against *Euglena* AsAP

	Monoclonal antibodies							
	EAP1	EAP2	EAP3	EAP4	EAP5	EAP6	EAP7	EAP8
Titration*	10^{-3}	5×10^{-6}	10^{-4}	7×10^{-1}	5×10^{-1}	5×10^{-2}	7×10^{-1}	5×10^{-2}
Subclass	IgG1	IgG1	IgM	IgM	IgM	IgM	IgM	IgM
Binding specificity	++	+++	+	+	+	+	+	+
Cross reactivity**								
S.sAsAP	++	—						
S.cAsAP	++	++						
K.cAsAP	++	++						
A.cAsAP	+	++						

+++,very strong; ++,strong; +,significant; — ,no effect
* The dilution of the supernatant fractions given 50% binding in the ELISA.
** S.sAsAP, spinach stromal AsAP; S.cAsAP, spinach cytosolic AsAP; K.cAsAP, komatsuna cytosolic AsAP; A.cAsAP, *Arabidopsis* cytosolic AsAP

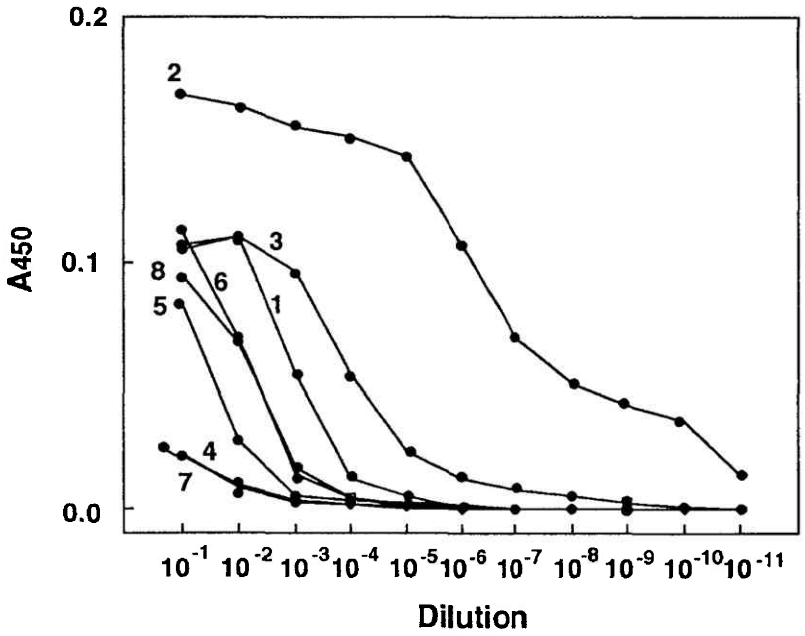

Fig. 2.7. An enzyme-linked immunosorbent assay of mAbs against *Euglena* AsAP. To 96-well microtiter plates precoated with *Euglena* AsAP, a dilution series of ascites were added. ELISA assay was performed as described in "Materials and Methods". 1-8, EAP1-EAP8.

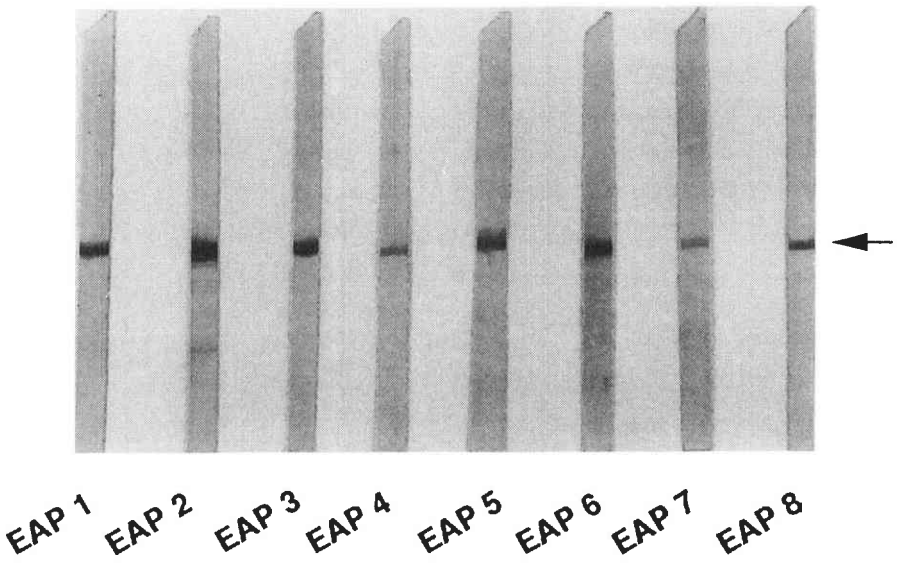

Fig. 2.8. Immunoblot analysis of *Euglena* AsAP with eight mAbs. Partial purifed AsAP (0.5 μg/lane) was subjected to SDS-PAGE on slab gels (10% polyacrylamide), electro-phoretically transferred onto a PVDF membrane, and reacted with EAP1 (lane 1), EAP2 (lane 2), EAP3 (lane 3), EAP4 (lane 4), EAP5 (lane 5), EAP6 (lane 6), EAP7 (lane 7), EAP8 (lane 8). Protein size markers were shown on the left. The arrow indicates the AsAP.

Cross reactivity of EAP1 and EAP2 with ascorbate peroxidase isozyme isolated from plant sources —— In order to examine the cross reactivity of the *Euglena* mAbs (EAP1 and EAP2), we performed immunoblot analysis of chloroplastic and cytosolic AsAP isozymes from several higher plants, which contain two more types of AsAP isozymes. On a DEAE-Sephacel column, the activities of AsAP isozymes designated P-I and P-II from spinach leaves were eluted as two separate peaks at 0.1 M and 0.14 M of KCl (Fig. 2.9). P-I was further purified to electrophoretic homogeneity. The molecular weight of the P-I was calculated to be 34 kDa as judged by gel filtration and SDS-PAGE, which value was in agreement with that of the tea stromal form (Chen and Asada 1989). The N-terminal amino acid sequence of the P-I was the following: YASDPAQLKNAREDIK-ELLQR (Fig. 2.3). This result exhibits a highly significant homology (75%) over the first 20 amino acid sequence of the N-terminus of tea stromal AsAP (Chen and Asada 1989). The P-I was particularly labile and showed a high donor specificity for AsA. In contrast, P-II was more stable than P-I and utilized pyrogallol at a higher rate than AsA as an electron donor, which agreed with the characteristics of the typical cytosolic AsAP described by Asada (1994). It turned out, therefore, that P-I is the stromal form and P-II is the cytosolic form. We also obtained cytosolic isozymes purified from komatsuna (*Brassica rapa*) (Chapter VI) and partially purifed from *Arabidopsis* in a manner similar to that described above.

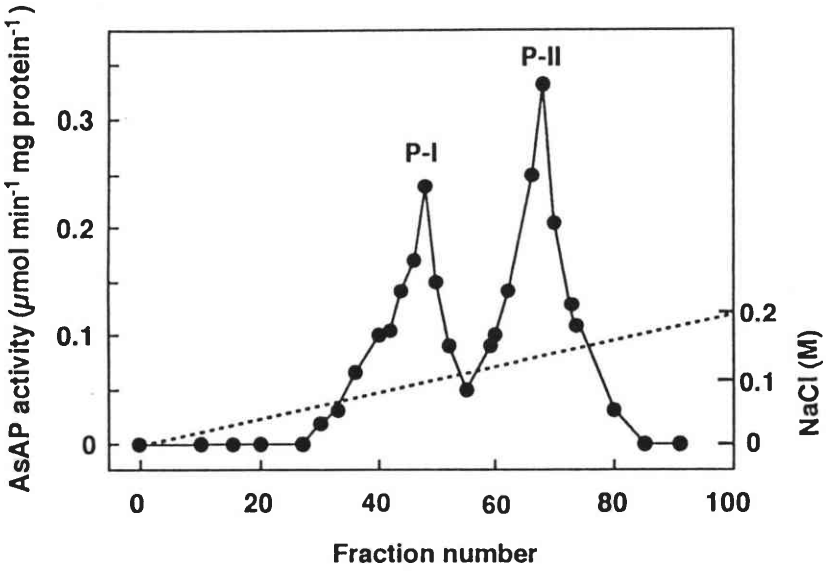

Fig. 2.9. Elution profile of spinach AsAP activities from the DEAE-Sephacel column. Detaile is described in the "Materials and Methods". (●) AsAP activity, (---) concentration of KCl.

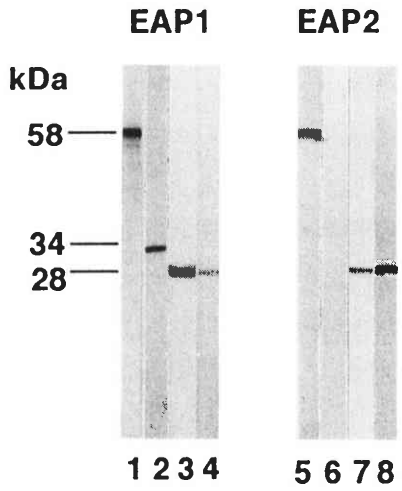

Fig. 2.10. Immunoblots of cytosolic and chloroplastic AsAPs in higher plants with EAP1 and EAP2. Each AsAP was subjected to SDS-PAGE and then electrotransferred to PVDF membranes, followed by immunoblotting with EAP1 and EAP2. Protein sizes are shown on the left.
Lanes 1 and 5, purified AsAP from *Euglena*; lanes 2 and 6, purified stromal AsAP from spinach; lanes 3 and 7, partially purified cytosolic AsAP from spinach; lanes 4 and 6, purified cytosolic AsAP from komatsuna (*Brassica rapa*).

As shown in Fig. 2.10, both EAP1 and EAP2 efficiently reacted with a 28 kDa band corresponding to the predicted size of the cytosolic AsAPs from spinach (P-II) and komatsuna. Both mAbs showed a cross-reaction with cytosolic AsAP from *Arabidopsis* but did not cross-react with guaiacol peroxidase from horseradish. These observations indicate that the *Euglena* AsAP is immunologically related to the higher plant cytosolic

and algal AsAPs. It is worth noting that EAP1 cross-reacted with both chloroplastic and cytosolic AsAP, suggesting that AsAP isozymes have a common epitope, that is, a highly antigenic region recognized by EAP1. It has been reported that antiserum raised against the tea stromal AsAP cross-reacted with both spinach thylakoid-bound AsAP and tea cytosolic AsAP (Chen and Asada 1989, Miyake et al. 1993). In contrast, the polyclonal antibodies to the pea cytosolic AsAP and the mAbs to spinach cytosolic AsAP failed to cross-react with the respective chloroplastic isozymes (Mittler and Zilinskas 1991, Saji et al. 1990).

In conclusion, the present results clearly reveal that the *Euglena* AsAP has both closely similar enzymlogical and immunological properties to those of AsAP isozymes from higher plants and its own properties (e.g., the reduction of lipid peroxides, the high molecular weight and the amino acid sequence of the N-terminus). In this regard, whether a similar type of AsAP exists in eukaryotic algae and cyanobacteria is an interesting problem. Oligonucleotide probes deduced from the peptide sequences and the mAbs from *Euglena* AsAP will be useful in exploring this question.

Summary

Asaorbate peroxidase has been purified to electrophoretic homogenity from *Euglena gracillis* Z. The enzyme showed a molecular mass of 58 kDa on SDS-PAGE and a gel filtration, indicating that *Euglena* asaorbate peroxidase exisits as a monomeric form. The substrate specificity for electron donor and the stabilty of the purified enzyme were similar to those of cytosolic isozymes from higher plants. One of the characteristic properties was that *Euglena* asaorbate peroxidase reduce organic hydroperoxides as well as H_2O_2. N-terminal amino acid sequence showed no significant similarity to any other asaorbate peroxidases from higher plants. However, the sequence of the peptides from the purified enzyme exhibited a high degree of homology to sequences of cytosolic and chloroplastic asaorbate peroxidases. Monoclonal antibodies against the purified *Euglena* asaorbate peroxidase were prepared. Two monoclonal antibodies (EAP1 and EAP2) showed high homology to cytosolic asaorbate peroxidases of higher plants, judged by western blot analysis. The EAP1 was also specific for chloroplastic asaorbate peroxidase from spinach. These findings indicate that *Euglena* asaorbate peroxidase exists in highly homologious regions with the asaorbate peroxidases of higher plants.

CHAPTER III

Effect of Iron on the Expression of Ascorbate Peroxidase in *Euglena*

Iron (Fe) serves as a prosthetic group for Fe-proteins, such as hemoglobin, cytochromes and guaiacol peroxidase (Aisen and Listowsky 1980). *Euglena* ascorbate peroxidase (AsAP) is also a hemoprotein, based on absorption spectra of the purified protein. In addition, spinach AsAP has contained a non-heme Fe, which is released from the enzyme in the AsA-depleted medium under aerobic conditions (Nakano and Asada 1987). These results suggest that Fe may regulate the expression of AsAP.

AsAP of *Euglena* and cyanobacteria, like glutathione peroxidase (GSHP) from animal sources can reduces organic hydroperoxides as well as H_2O_2 (Shigeoka et al. 1980b, Chapter II), suggesting that AsAP protects the cell membrane and cell-bound enzymes from the damage of the integrity and the inactivation, by the reduction of lipids peroxides generated *in vivo*.

This chapter reports the requirement and uptake of Fe by *Euglena* cells and the effect of Fe on the *Euglena* AsAP activity in order to find a clue to the expression mechanism of AsAP by Fe. Finally, I determine lipid peroxides in Fe-sufficient and -deficient cells and discuss the important physiological function of AsAP with regard to the lipid peroxide-scavenging system.

Materials and Methods

Organism and culture —— *Euglena gracilis* z (Fe-sufficient cells) were grown organotrophically at 26°C for 5 days under illumination (55 μE m^{-2} sec^{-1}) in 150 ml of Koren-Hutner medium containing 50 mg l^{-1} ferrous ammonium sulfate which corresponded to 7.1 mg l^{-1} (0.13 mM) of Fe as describe in Chapter II. The cells (14 × 10^6 cells ml^{-1}) grown in this manner were transferred to a basal medium (150 ml) lacking Fe and cultured for 5 days. Subsequently, the cells (1 ml) in stationary phase were again cultured in a Koren-Hutner medium lacking Fe for 5 days to obtain Fe-deficient cells (9 × 10^6 cells ml^{-1}). Fe-sufficient cells were grown for 5 days in a medium supplemented with 30-, 60- and 100-fold higher concentration of Fe in comparison with that of the original Koren-Hutner medium. These cells, in the stationary phase, represent Fe-excess cells. Cell number was determined with a haemocytometer.

Determination of iron —— *Euglena* cells (wet wt. 0.2 g) were harvested by centrifugation at 3000 × g for 5 min, washed twice with distilled water and resuspended in 5 ml of nitric acid. The concentration of Fe was assayed by measuring absorbance of

Fe at 2493Å using an atomic absorption spectrophotometer (Shimadzu AA-640-12). The relation of peak height to the quantity of Fe was linear up to 100 μmol.

Assay of iron uptake —— Fe-deficient cells grown for 5 days, by which time the stationary phase was reached, was used for assay of iron uptake. 0.13 mM Fe was added to 50 ml of cell culture of Fe-deficient cells. At given intervals, a 5 ml sample was withdrawn and centrifuged at 1000 × g for 5 min to obtain the cell and supernatant fractions. The content of Fe in both fractions was determined as described above.

Subcellular distribution of iron —— Fe-deficient *Euglena* cells (wet wt. 0.2 g), which had taken up exogenous Fe for 2 h, were disintegrated by sonication (10 kHz, 2 min) in 3 ml of 50 mM Tris-HCl buffer (pH 6.9) and centrifuged at 500 × g for 3 min to remove the cell debris. The cell homogenate was centrifuged at 10000 × g for 10 min to obtain the 10000 × g-precipitate and then the supernatant fraction was ultracentrifuged at 100000 × g for 30 min. Subsequently, the 100000 × g-supernatant fluid was chromatographed on a Sephadex G-25 column (1.8 × 45 cm) equilibrated with 50 mM Tris-HCl buffer (pH 6.9) at a flow rate of 24 ml h^{-1} and the eluate was collected (1.2-ml fraction).

Assays of ascorbate peroxidase and lipid peroxides —— Fe-sufficient, -deficient and -excess *Euglena* cells grown in the stationary phase were used for assays of AsAP activity and lipid peroxides. Crude extracts of *Euglena* cells were prepared and assayed for AsAP as described in Chapter II. Lipid peroxidation was assessed by measurement of thiobarbituric acid-reactive substances (TBARS) (Buege 1978). Five ml of 1% (w/v) trichloroacetic acid was added to *Euglena* cells (wet wt. 1 g). The mixture was sonicated (10 kHz) for a total of 1 min with two intervals of 30 s each and centrifuged at 10000 × g for 10 min. The supernatant fraction was used to determine TBARS. Each value represents the mean of four assays ± S.D.

Immunoblotting —— Proteins were separated using SDS-PAGE and blotted onto an Immobilon-P transfer membrane (PVDF, pore size 0.45 μm, No. IPVH 304FO, Millipore, Bedford, MA) using a semidry electroblotting system according to the manufacturer's instructions (Bio-Rad). Immunoblotting was performed according to the method of Chapter II. *Euglena* monoclonal antibodies were prepared as described in Chapter II.

Results and Discussion

Requirement and uptake of iron by Euglena *cells* —— Fig. 3.1 shows growth curves of Fe-sufficient, -deficient and -excess (30-fold) *Euglena* cells. Each culture reached stationary phase in 5 days. The cell growth of Fe-deficient cells decreased to 66% in comparison with that of Fe-sufficient cells.

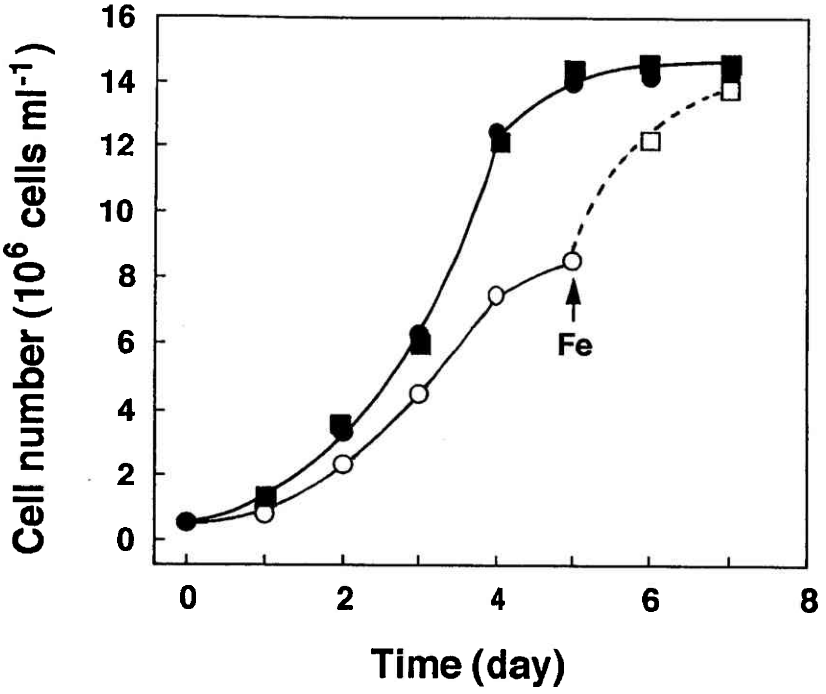

Fig. 3.1. Growth curves of Fe-sufficient, -deficient and-excess *Euglena* cells. ●, Fe-sufficient; ○, Fe-deficient; ■, Fe-excess (30-fold). The solid line (□) shows the growth curve of Fe-deficient cells after the addition of 7.1 mg l^{-1} Fe. Each experimental point represents the mean of four assays (coefficient of variation <5%).

When 0.13 mM Fe, which corresponds to that of Fe-sufficient cells, was added to Fe-deficient cultures, cell division commenced and the cell number reached the same level as that of Fe-sufficient cells within 2 days. The content of Fe in *Euglena* cells in stationary phase under each growth condition was determined. Fe-sufficient cells contained 0.18 ± 0.02 mg of Fe per 10^9 cells, indicating that 35.5% of the amount of Fe present in the original medium was taken up in the Fe-sufficient cells. In contrast, Fe was not detected in Fe-deficient cells grown for 4 to 6 days, showing that Fe-deficient cells contain little iron or extremely low amounts beyond the limit of the Fe measurement. These results demonstrate that Fe is absolutely required for the growth of *Euglena* cells. When cells were grown in a medium supplemented with a 30-fold higher Fe concentration (213 mg l^{-1}) than that of Fe-sufficient cells, there was no change of the cell growth in a stationary phase. The same result was obtained from cells grown in a medium containing 60- or 100-fold higher Fe concentration. The cellular content of Fe in 30-, 60- and 100-fold Fe-excess cells was 0.38 ± 0.02, 0.78 ± 0.03 and 1.13 ± 0.03 mg, respectively, per 10^9 cells, indicating that 2.7%, 2.7% and 2.4% of the original amount of Fe supplied was accumulated in the cells. These results suggest that *Euglena* possesses a regulatory system that prevents the incorporation of a large amount of external Fe so that the cellular concentration of Fe is maintained at relatively low levels.

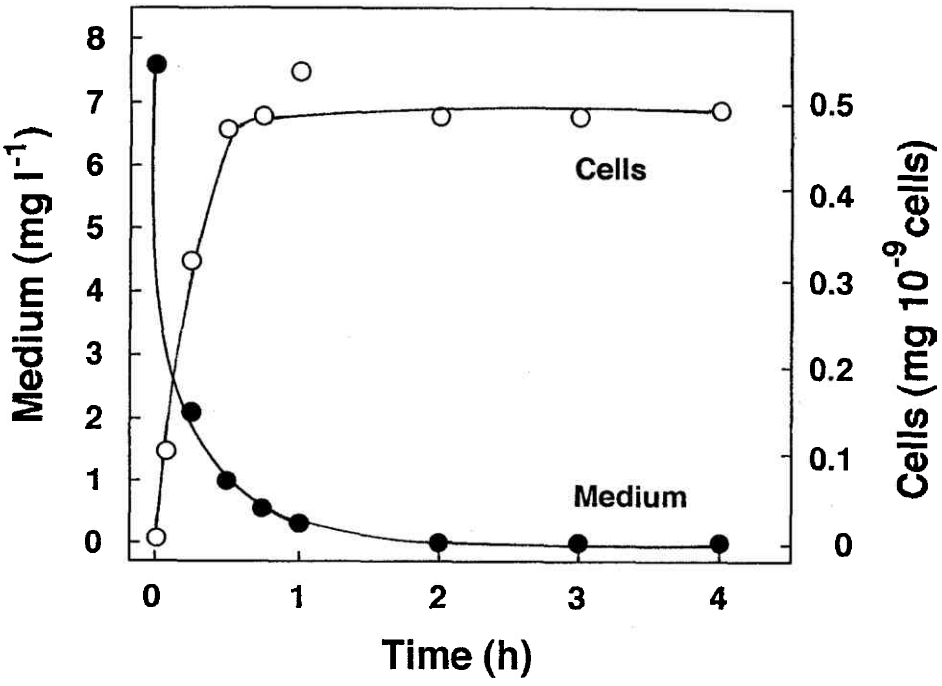

Fig. 3.2. Changes in Fe levels in Fe-supplemented cells and medium. ○, Fe in cells; ●, Fe in medium. 0.13 mM Fe was added to the Fe-deficient cells.

When 0.13 mM Fe was added to Fe-deficient cultures, Fe was incorporated linearly and peaked at 1 h (Fig. 3.2). The rate of Fe uptake in Fe-deficient cells was calculated as 2.5 μmol h^{-1} 10^{-9} cells. The subcellular distribution of Fe taken up for 2 h was examined by the differential centrifugation method (Table 3.1). Of Fe, 69% was located in 10000 × g-precipitated fraction, 4% in 100000 × g-precipitated fraction and 18% in 100000 × g-supernatant fraction. Subsequently, the 100000 × g-supernatant was chromatographed on a Sephadex G-25 column. As shown in Fig. 3.3, Fe was predominantly eluted in the high molecular weight fractions. These data demonstrate that Fe incorporated into the *Euglena* cells exists mostly in a bound form, not in a free form. It has been reported that free Fe becomes toxic to many cellular components since it is involved in the reaction of superoxide anion and hydrogen peroxide, that is, the Fenton reaction, to produce hydroxyl radicals which are the most reactive species of active oxygen (Halliwell and Gutterridge 1985). In V79 Chinese hamster ovary cells, oxidative stress induces activation of a cytosolic protein, named iron-responsive element-binding protein, responsible for control of Fe uptake (Martins et al. 1995). It seems to be a forthcoming problem whether a protein related to regulation of Fe uptake exists in *Euglena* cells. Accordingly, the facts that *Euglena* cells limit the cellular level of Fe and avoid

accumulation of free cellular Fe seem to be a general strategy for the suppression of the oxidative stress produced by free Fe.

Table 3.1. Distribution of Fe content and protein in subcellular fractions of Fe-deficient cells with added 0.13 mM Fe for 2 h.

Fraction	Protein (μg)	Fe content (μg)
Crude homogenate	190.0 ± 6.8 (100%)*	65.0 ± 5.1 (100%)
10,000 × g Ppt	92.0 ± 5.6 (46)	44.7 ± 3.5 (69)
100,000 × g Ppt	24.8 ± 2.1 (13)	2.9 ± 0.2 (4)
100,000 × g Sup	51.5 ± 4.5 (27)	11.5 ± 1.8 (18)

Preparation of crude homogenate and differential centrifu-gation was carried out as described in "Materials and Methods". Each value represents the mean of three assays ± S.D. *Percent distribution in parentheses.

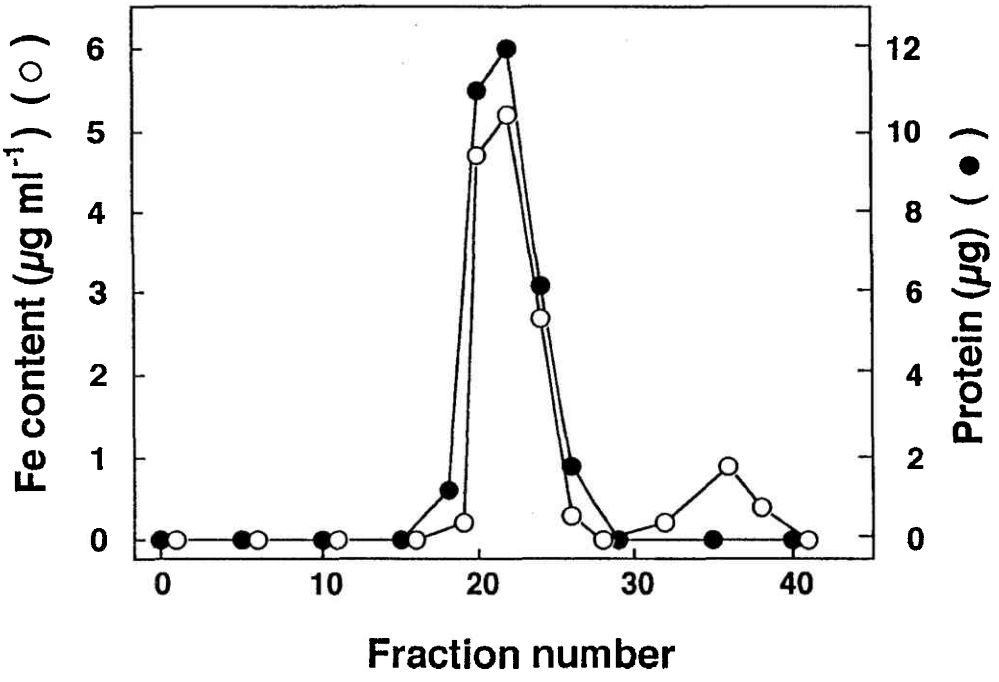

Fig. 3.3 Elution pattern of Fe in the 10000 × g-supernatant fraction by column chromatography on Sephadex G-25. The 10000 × g-supernatant fraction was obtained by differential centrifugation was applied to a Sephadex G-25 column and eluted with a 50 mM Tris-HCl buffer (pH 6.9). See Materials and Methods for details. ○, Fe; ●, Protein.

Effect of iron on the Euglena *ascorbate peroxidase activity*——It is well known that Fe plays a critical role in the expression of heme and non-heme proteins of living organisms (Aisen and Listowsky 1980). AsAP is a hemoprotein like guaiacol peroxidase and cytochrome *c*. By dialysis of the purified spinach AsAP against 50 mM phosphate buffer (pH 7.6), the Fe content of the enzyme decreased to about half and the activity was

lost, because AsAP also contains one atom of non-heme Fe, which is involved in the stability of the enzyme (Nakano and Asada 1987, Chen and Asada 1989). In this context, it is interesting to investigate how Fe affects the activity of AsAP in Fe-sufficient and -deficient *Euglena* cells. Fe-sufficient cells in the stationary phase contain 75.3 ± 2.3 μmol min^{-1} 10^{-9}cells of AsAP activity, which corresponds to the value reported previously (Shigeoka et al. 1987c) (Table 3.2). To my surprise, no enzyme activity was found in Fe-deficient cells grown for 4 to 6 days. Shigeoka et al. (1980a) have previously reported that 50 μM ferrous sulfate is necessary to prevent extractable AsAP from inactivation. However, the enzyme activity was not recovered by incubation of the crude extract prepared from Fe-deficient cells with 50 μM ferrous sulfate. These results suggest that Fe fed exogenously is incorporated into *Euglena* cells and then is involved in the expression of AsAP activity. Radtke et al. (1992) showed that the proliferation of Fe-deficient *Euglena* cells decreased by the addition of 100 μM H_2O_2, but Fe-sufficient cells had no effect. This result may account for the insufficiency of AsAP in Fe-deficient cells.

Table 3.2. Effect of iron on AsAP and lipid peroxides in *Euglena*. Fe-sufficient, -deficient and -excessive *Euglena* cells grown in the stationary phase were used for assays for AsAP activity and lipid peroxides (TBARS) as described in "Materials and Methods".

Cells	Ascorbate peroxidase activity (μmol min^{-1} 10^{-9}cells)	TBARS (μmol 10^{-9}cells)
Fe-sufficient	75.3 ± 2.3	6.2 ± 0.6
Fe-deficient	n.d.	15.8 ± 2.1
Fe-excessive	77.5 ± 3.1	14.3 ± 1.9

Each value represents the mean of four assays ± S.D.
n.d.: not detected.

As mentioned above, AsAP is a hemoprotein and the enzyme activity was not detected in Fe-deficient cells, suggesting that the Fe-deficient cells may be used for elucidating the effect of Fe on the AsAP protein synthesis. The addition of 1500 mg l^{-1} ferrous ammonium sulfate to the Fe-deficient cells causes the AsAP activity to rapidly increase over 3 h and then linearly up to 24 h (Fig. 3.4). The simultaneous addition of cycloheximide (CHI; 5 × 10^{-4} M), an inhibitor of protein synthesis, has no effect on the increase in the AsAP activity over 3 h, whereas the increase in the activity from 3 to 24 h is completely inhibited by CHI. When CHI was added to culture medium at different times, the increase in AsAP activity was completely inhibited (Fig. 3.4). On immunoblot analysis using anti-*Euglena* AsAP monoclonal antibody, the AsAP protein (58 kDa) is

detected in the Fe-deficient cells. The level of AsAP protein does not change up to 3 h, but increases in parallel with the increase in the AsAP activity up to 24 h (Fig. 3.5). These results suggest that the rapid increase in AsAP over 3 h by Fe is due to the activation of pre-existing protein and the increase from 3 to 24 h is attributable to the protein synthesis of AsAP. Succinyl acetone, an inhibitor of heme synthesis, completely inhibits the increase in AsAP activity from 3 to 24 h, but not the increase in the enzyme activity up to 3 h (Fig. 3.6). This result and the inhibitory effect of cycloheximide (Fig. 3.4) suggest that the syntheses of heme and protein of AsAP may be involved in the increase in AsAP activity from 3 to 24 h. In *Escherichia coli*, the Fur protein that is activated through binding to Fe^{2+}, serves as a transcriptional regulator of *sod*A (coding for MnSOD) and *sod*B (FeSOD) (Fee 1991). The transcription factor MAC1 of yeast which regulate the *CTT1* gene (coding for cytosolic catalase) is necessary for the basal transcription level of *FRE1* (coding for the transmembrane Fe^{3+} reductase) and for the feedback regulation by elevated Fe (Jungmann et al. 1993). Isolation of cDNAs encoding the *Euglena* AsAP will be a useful tool for investigating AsAP gene expression by the addition of Fe.

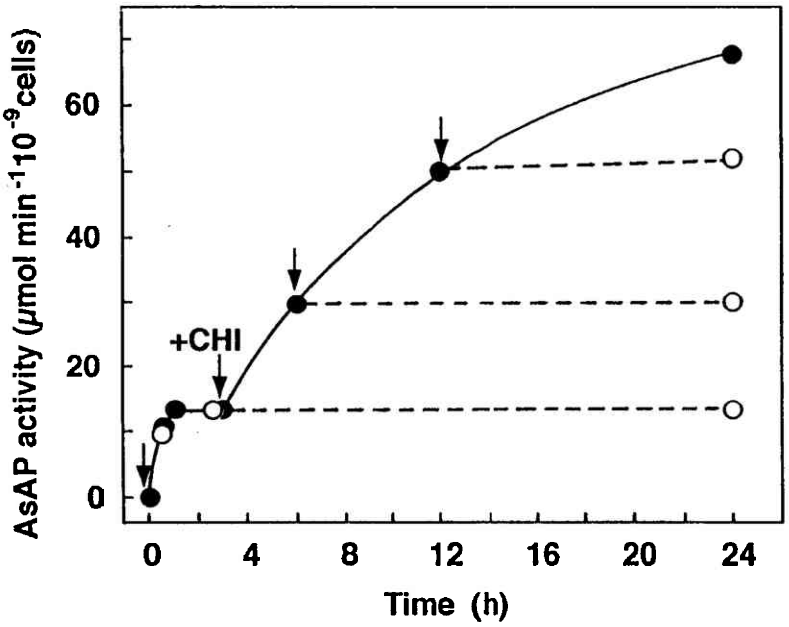

Fig. 3.4. Effect of Fe and cycloheximide (CHI) on AsAP activity in Fe-deficient cells. 1500 mg l^{-1} ferrous ammonium sulfate was added to Fe-deficient cells. Each arrow represents the addition of CHI (5×10^{-4} M). Solid line shows the activity of AsAP after the addition of CHI. *Euglena* cells (wet.wt 0.2 g) were harvested by centrifugation (500 × g), washed twice with deionized water, resuspended in 50 mM potassium phosphate buffer, pH 6.3, containing 1 mM AsA, and disintegrated by sonication (10 kHz) for 2 min. The lysate was centrifuged at 10000 × g for 10 min, and the supernatant was used as a crude extract. AsAP activity was assayed as described "Materials and Methods".

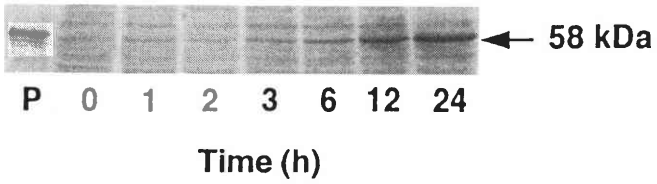

Fig. 3.5. Immunoblot analysis of AsAP protein levels in Fe-supplemented *Euglena* cells. The crude protein (50 μg) from Fe-deficient and -supplemented *Euglena* cells were separated by SDS-PAGE, blotted to PVDF membrane, and detected with *Euglena* AsAP monoclonal antibodies as described in "Materials and Methods". A arrow represents AsAP protein, which corresponding to a molecular mass of 58 kDa. P; purified *Euglena* AsAP.

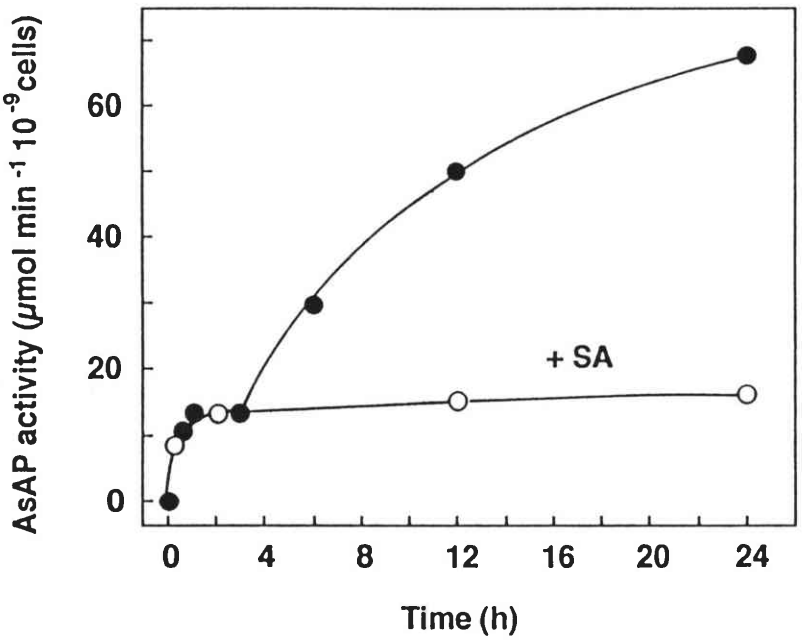

Fig. 3.6. Effect of succinyl acetone (SA) on AsAP activity of Fe-deficient *Euglena* cells. 5 mM SA was added to Fe-deficient cells, 1 h before addition of 1500 mg l^{-1} ferrous ammonium sulfate. See the legend of Fig. 3.4. for a full account of detailed procedures for the experiments.

Detection of Lipid peroxide —— AsAP in *Euglena* (Shigeoka et al. 1980a, chapter II) and some cyanobacteria (Tel-Or et al. 1986) reduces various organic hydroperoxides as well as H_2O_2, suggesting that this enzyme, like GSHP, protects the cell membrane and prevents inactivation of membrane-bound enzymes from damage by lipid peroxides generated endogenously from unsaturated fatty acids. *Euglena* contains a large quantity of polyunsaturated fatty acids (Hulanicka et al. 1964). As mentioned above, the AsAP activity was not observed in Fe-deficient cells. These results indicate that Fe-deficient *Euglena* cells may be useful organisms for elucidating in detail the physiological role of

AsAP in the lipid peroxide-scavenging system. Thus TBARS was determined in Fe-sufficient, -deficient and -excess *Euglena* cells grown to stationary phase (Table 3.2). TBARS in Fe-deficient cells was about 2.5-fold greater than that in Fe-sufficient cells. The increase in TBARS in Fe-deficient cells seems to stem from the depletion of AsAP activity. The TBARS in Fe-excess cells was 2.3-fold higher than that in Fe-sufficient cells. In this case, AsAP may be unable to remove large amounts of lipid peroxides from lipid peroxidation, caused by hydroxyl radicals produced by Fe accumulated in Fe-excess cells. The findings that the activity of AsAP in Fe-excess cells did not increase with the cellular concentration of Fe (Table 3.2) and that 73% of Fe incorporated into the cells was distributed in the cell membranes (Table 3.1) support this view. Thus these facts suggest that *Euglena* AsAP possesses a lipid peroxide scavenging function as a second important function, next to its action of destroying H_2O_2 *in vivo*.

Summary

Euglena gracilis shows an absolute requirement for iron for growth. Iron-deficient cells exhaustively take up iron within 1 h of the addition of iron. Incorporated iron exists in a bound form, but not in a free form. The ascorbate peroxidase activity is not found in iron-deficient cells. The addition of iron to the iron-deficient cells causes the ascorbate peroxidase activity to rapidly increase over 3 h and then linearly up to 24 h. Immunoblot analysis and inhibitory effect of cycloheximide and succinyl acetone suggest that the syntheses of heme and protein of ascorbate peroxidase may be involved in the increase in ascorbate peroxidase activity from 3 to 24 h. Lipid peroxides (thiobarbituric acid-reactive substances) in iron-deficient cells is much higher than those in iron-sufficient cells. These results suggest that iron is involved in the expression of ascorbate peroxidase and *Euglena* ascorbate peroxidase may possess a second important function in the form of a lipid peroxide scavenging system, in addition to its action of destroying hydrogen peroxide.

CHAPTER IV

Effect of Illumination on the Induction of Ascorbate Peroxidase and Enzymes Related to Ascorbate-Glutathione Cycle in *Euglena gracilis* Z

Environmental stresses such as high light intensity (Gillham and Dodge 1987), low temperature (Jahnke et al. 1991), and drought (Mittler and Zilinskas 1994, Sgherri and Navari-Izzo 1995) have been correlated with increased production of active oxygen species and activity of antioxidant enzymes. It has been known, for instance, illuminated chloroplasts produce H_2O_2 by electron transfer from photosystem I to O_2 to form superoxide (O_2^-) from which H_2O_2 is formed by the action of superoxide dismutase (SOD) (Mehler 1951, Asada 1994). In *Euglena* cells, Tschiersch and Ohmann (1993) observed the formation of reactive oxygen in the process of photoinhibition related to photosystem II and thylakoids.

Shigeoka et al. (1979b, 1987d) have reported that when dark-grown *Euglena* cells were illuminated, the cellular content of AsA and GSH increased by about 7- and 4.5-fold, respectively. These results are to be expected for the response of the enzymes related to AsA-GSH cycle including ascorbate peroxidase (AsAP) to light condition.

The present chapter reports that illumination increases the activity of AsAP and the enzymes related to AsA-GSH cycle (SOD, monodehydroascorbate [MDAsA] reductase, dehydroascorbate [DAsA] reductase, and GSH reductase) and that the increases are due to de novo synthesis of thier protein.

Materials and Methods

Organism and culture conditions——*Euglena gracilis* Z was maintained at 26°C in the dark condition and precultured heterotrophically in the KH medium as described in Chapter II. Cell number was determined with a haemocytometer.

Preparation of crude extract——*Euglena* cells were harvested by centrifugation, washed twice, resuspended in 50 mM potassium phosphate buffer, pH 6.3, containing 10% (w/v) sucrose, 1 mM EDTA, and 1 mM AsA and disintegrated by sonocation (10 kHz) for 2 min. The lysate was centrifuged at 10000 × g for 10 min, and the supernatant was used as a crude extract.

Enzyme assays——The activity of AsAP was determined spectrophotometrically as described in Chapter II.

SOD was assayed spectrophotometrically as the inhibition of xanthine oxidase-dependent reduction of 10 μM ferricytochrome *c* (Sigma, U.S.A.) monitored at 550 nm in 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, and 50 μM xanthine. One

unit of SOD inhibited by 50% a control rate, established by sufficient xanthine oxidase, of ferricytochrome *c* reduction of 0.025 A_{550} min^{-1} (Kanematsu and Asada 1979)

MDAsA for the MDAsA reductase assay was generated by AsA and an AsA oxidase system according to Arrigoni et al. (1981). The reaction mixture (2 ml) contained 50 mM phosphate buffer (pH 7.0), 1 mM AsA, 1 unit AsA oxidase (Boehringer Mannheim, Germany), 0.2 mM NAD(P)H, and enzyme and the reaction was started by adding AsA oxidase.

DAsA reductase was assayed in a reaction mixture (2 ml) containing 50 mM phosphate buffer (pH 7.0), 2.5 mM DAsA, 2.5 mM GSH, 1 unit GSH reductase (Sigma, U.S.A.), 0.2 mM NADPH, and enzyme by measuring the decrease in absorbance of NADPH at 340 nm, 6.22 mM^{-1} cm^{-1} (Shigeoka et al. 1987c).

GR was assayed in 2 ml of reaction mixture containing 50 mM phosphate buffer (pH 8.2), 1 mM EDTA, 0.2 mM NADPH, and 0.2 mM GSSH and the enzyme as described previously (Shigeoka et al. 1987a). The progress of the reaction was monitored by measuring the decrease in absorbance of NADPH at 340 nm (6.22 mM^{-1} cm^{-1}).

Immunoblot analysis —— Western immunoblotting using monoclonal antibodies (mAbs) raised against purified *Euglena* AsAP was carried out by the method of described in Chapter II. The crude extracts were subjected to SDS-PAGE on 10% slab gel. Following SDS-PAGE, gels were equilibrated for 20 min in transfer buffer (25 mM Tris, 192 mM glysine and 20% [v/v] methanol). Proteins were transferred electropho-retically to a PVDF membrane by using a semi-dry blotting apparatus (Bio-Rad). Immunodetection of proteins on blots was done at room temperature with 0.1% BSA in PBS buffer as a blocking reagent. Peroxidase-conjugated goat anti-(mouse Igs) serum used as a secondary antibody.

Protein assay —— Protein was determined by the method of Bradford (1976) with BSA as a standard.

Results and Discussion

Effect of illumination on ascorbate peroxidase activity and enzyme activities related to AsA-GSH cycle —— To investigate the possible light-dependence of the expression of the AsAP activity, *Euglena* cells were subjected to a light shift treatment in which dark-grown cells were switched to different light-conditions. Fig. 4.1 shows the effect of illumination on the AsAP activity in the dark-adapted *Euglena* cells. When cultured in the dark condition, *Euglena* cells contained AsAP activity equivalent to 25 nmol min^{-1} 10^{-6}cells. Illumination at 55 μE m^{-2} sec^{-1} allowed approximately 3.5-fold increase in the enzyme activity up to 24 h. The same result was obtained by illumination at 150 μE m^{-2} sec^{-1}. When the cells were illuminated at 20 μE m^{-2} sec^{-1}, the AsAP activity increased

gradually up to 72 h to reach a same level of illumination at 55 μE m^{-2} sec^{-1}. These results suggested that the increase in the AsAP activity is saturated with a light intensity of 55 μE m^{-2} sec^{-1}.

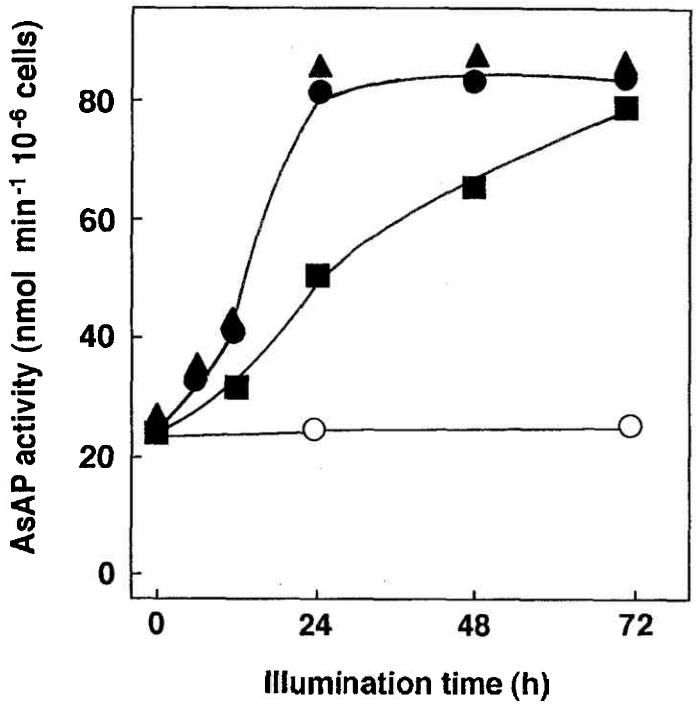

Fig. 4.1. Effects of illumination on AsAP activities in *Euglena* gracilis. ■, 20 μE m^{-2} sec^{-1}; ●, 55 μE m^{-2} sec^{-1}; ▲, 150 μE m^{-2} sec^{-1} Each data point represents the mean of three replicates.

Euglena cells contain two Fe-SOD in soluble fraction and a Mn-SOD in thylakoids (Kanematsu and Asada 1979). Furthermore, all the enzymes related to AsA-GSH cycle are present in the cytosol, not in the chloroplasts (Shigeoka et al. 1987a, 1987c). These facts suggest that the active oxygen scavenging enzymes are effectively operative as a conjugated system in response to light intensity. In addition to AsAP activity, the increase in SOD activity and enzyme activities related to AsA-GSH cycle were obserbed (Fig. 4.2). SOD activity rose 5- to 6-fold after 8 h of illumination at 150 μE m^{-2} sec^{-1} and declined during the remainder of the illumination priod. The activities of MDAsA reductase, DAsA reductase, and GSH reductase were found to rise approximately 3-, 4-, and 8-fold, respectively, after 24 h of illumination at 150 μE m^{-2} sec^{-1}. Increases in activities of AsA-GSH cycle enzymes in illuminated *Euglena* cells are accompanied by the increase in AsAP activity.

Previously, Shigeoka et al. (1979b) have reported that illumination of dark-grown *Euglena* cells for 4h at 35 μE m^{-2} sec^{-1} causes about a 7-fold increase of the cellular content of AsA. Moreover, when dark-grown *Euglena* cells were illuminated at 55 μE m^{-2} sec^{-1}, the content of total GSH increased by about 4.5-fold to reach a peak after 7 h (Shigeoka et al. 1987d). The light-dependent increase of AsA-GSH cycle enzymes,

related to the adaptation of the *Euglena* cells to the illumination, is also closely connected with those of AsA and GSH. These facts clearly indicate that elevated levels of antioxidative components can be considered an early physiological response of *Euglena* cells to remove H_2O_2 generated by illumination.

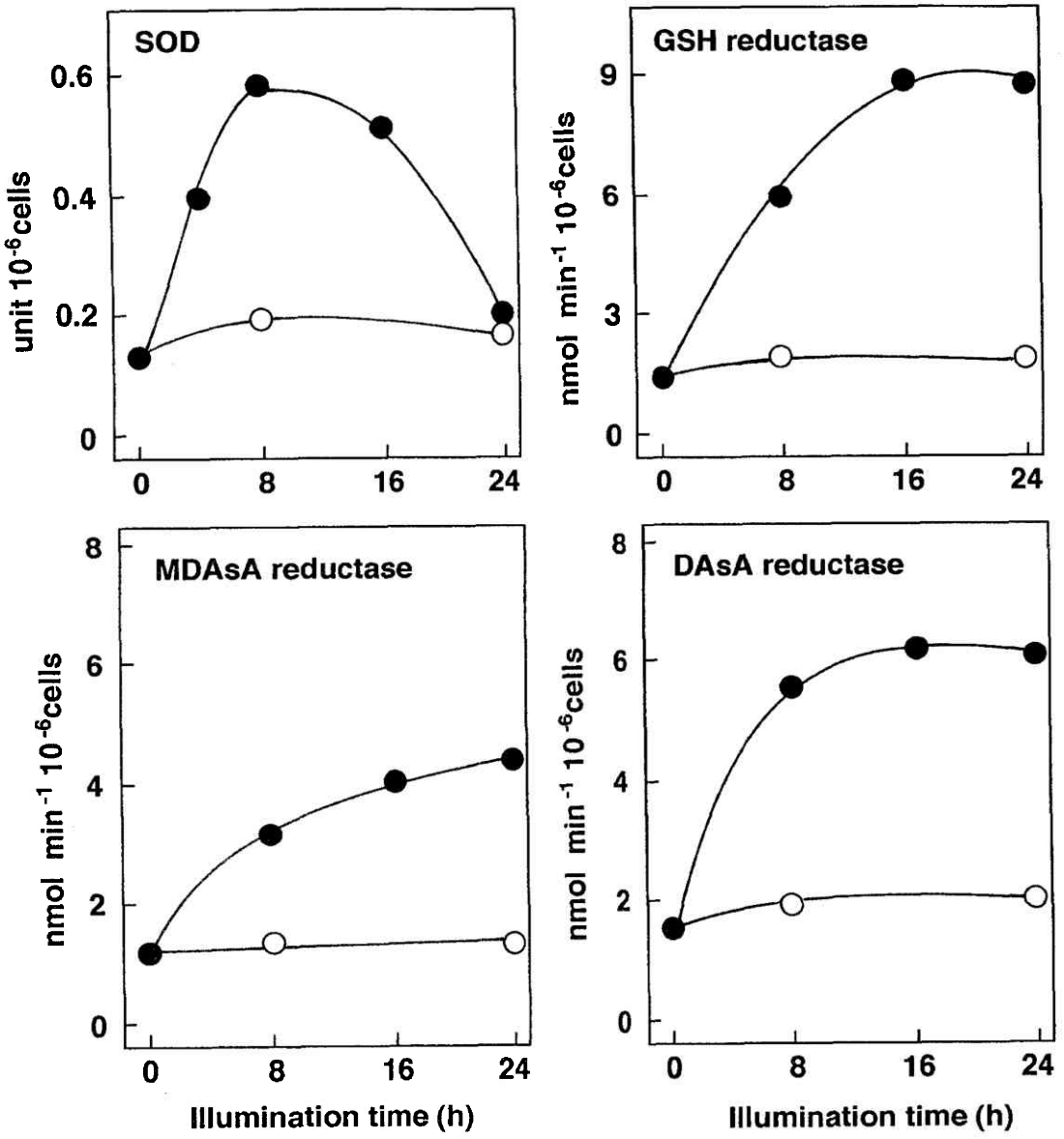

Fig. 4.2. Effect of illumination at 150 μE m^{-2} sec^{-1} on SOD activity and enzyme activities related to AsA-GSH cycle. ●, enzyme activities ○, enzyme activities after addition of cycloheximide. Cycloheximide was added in the dark to the culture medium at a final concentration of 5×10^{-5} M, which did not affect cell viability. Each data point represents the mean of three replicates.

In higher plants, report in the literature showed chloroplasts isolated from pea leaves grown at a higher light intensity (400 μmol m^{-2} sec^{-1}) contained enhanced activities of AsAP, GSH reductase and DAsA reductase, and higher levels of AsA than chloroplasts grown at a lower light intensity (100 μmol m^{-2} sec^{-1}) (Gillham and dodge

1987). Thomsen et al. (1992) reported that the appearance of AsAP is regulated by light via phytochrome. Furthermore, recent studies reported that in higher plants, high light intensity, especially in combination with chilling, magnesium deficiency treatment and high temperature, increases the levels of AsA and activities of antioxidant enzymes including AsAP (Schöner and Krause 1990, Cakmak and Marschner 1992, Fuse et al. 1993). These results indicate that light is a important factor in the regulation of active oxygen scavenging systems in photosynthetic organisms.

Effects of some antibiotics on ascorbate peroxidase and enzymes related to AsA-GSH cycle ——Effects of some antibiotics on the activity of AsAP in *Euglena* cells after 24 h in the light are shown in Table 4.1. Cycloheximide (CHI), an inhibitor of protein synthesis on the 87S cytoplasmic ribosomes (Bovarnick et al. 1974a, 1974b), completely inhibited the increase in AsAP activity, whereas chloramphenicol and streptomycin, specific inhibitors of protein synthesis on the 68S plastid ribosomes (Bovarnick et al. 1974a, 1974b), had little effect. When CHI was added to the medium at different stages during the cell cycle, AsAP activity promptly stopped. CHI also inhibited the increase in SOD, MDH reductase, DAsA reductase, and GSH reductase activities (Fig. 4.2). These results showed that the increase in activities of AsA-GSH cycle enzymes including AsAP are attributable to a photoinduction of de novo protein synthesis on cytoplasmic ribosomes.

Table 4.1. Effects of some antibiotics on the increase of AsAP activity in *Euglena* gracilis on illumination. Antibiotics were added in the dark to the culture medium at a final concentration shown, which did not affect cell viability. Effects are shown as percentage inhibition after 24 h as compared with the increase of the AsAP activity in the control to which no antibiotic was added.

Antibiotics	Concentration (M)	Inhibition (%)
Cycloheximide	5×10^{-5}	100
Chloramphenicol	3×10^{-3}	7.5
Streptomycin	10^{-5}	1.5

Effect of illumination on ascorbate peroxidase protein translation levels—— Although several studies have demonstrated an increase in AsAP activity in response to light intensity, no informations about the molecular mechanism including protein translation have existed. In order to examine the relationship between the light-dependent induction of AsAP activity and the translation of AsAP protein, immunoblot analysis using monoclonal antibodies (mAbs) raised against purified *Euglena* AsAP (Chapter II) was performed. *Euglena* cells were harvested after different periods of exposure to light (55

μE m^{-2} sec^{-1}) and total cellular protein was resolved by SDS-PAGE, with gel lanes loaded on the basis of equal proteins. A 58 kDa protein bands corresponding to the purified AsAP was detected. Fig.4.3. showed that the accumulation of AsAP protein increased with the elapse of time. The accumulation of AsAP protein was changed in parallel to that of the AsAP activity depending on the presence of illumination. These results suggest that under the light stress, AsAP contributes to the defense system by the increasing levels of AsAP activity depending on the de novo protein synthesis of AsAP protein.

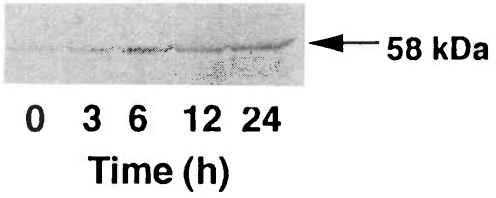

Fig. 4.3 Immunoblot analysis of AsAP protein prepared from illuminated *Euglena* cells. Total soluble protein was extracted from *Euglena* cells, resolved by SDS-PAGE, and electroblotted to PVDF membrane as described in "Materials and Methods". Each lane contained 100 μg of soluble protein. *Euglena* cells were illuminated at 55 μE m^{-2} sec^{-1} for 0, 3, 6, 12, and 24 h.

In higher plants, the situation is often considerably complicated by the presence of a large number of isoenzyme forms; for example, the large GSH reductase and SOD families of isoenzymes, encoded by different genes (Foyer et al. 1994). AsAP is also located in chloroplasts and cytosol (Asada 1994). It is important to consider the approach to understand the response to environmental stresses may be the isolation and engineering of regulatory genes that control the synthesis of different enzymes involved in oxidative stress response. At the point of this view, it is anticipated that the *Euglena* cells would be useful species to disolve this problem, because all the enzymes related to the AsA-GSH cycle located only in the cytosol, not in the organelles (Shigeoka et al. 1987a, 1987c, Chapter II). It is interesting problem why the *Euglena* cells lack AsAP from chloroplasts and how the cells are functioning to scavenge H_2O_2 generated in chloroplasts. This problem will be discussed in Chapter V.

Summary

Illumination of dark-grown *Euglena* cells for 24 h at 55 μE m^{-2} sec^{-1} caused about 3.5-fold increase in the ascorbate peroxidase activity. The increase in the ascorbate peroxidase activity was saturated with a light intensity of about 55 μE m^{-2} sec^{-1}. The activities of enzymes involved in ascorbate-glutathione cycle, superoxide dismutase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase

were also found to increase during illumination at 150 μE m^{-2} sec^{-1}. Increases in activities of these enzymes in illuminated *Euglena* cells are accompanied by the increase in ascorbate peroxidase activity. The inductions of the enzyme activities related to ascorbate-glutathione cycle including ascorbate peroxidase by illumination were inhibited by cycloheximide, but not chloramphenicol and streptomycin. Immunoblot analysis showed the accumulation of ascorbate peroxidase protein was changed in parallel to that of the ascorbate peroxidase activity depending on the intensity of illumination. These results clearly suggest that the increased level of the enzyme activities in ascorbate-glutathione cycle is attributed to synthesis de novo of their proteins.

CHAPTER V

Hydrogen Peroxide Generation in Organelles of *Euglena gracilis* Z

H_2O_2 is generated by the following two reactions; one is the univalent reduction of oxygen to form superoxide radicals followed by a disproportionation with enzyme or non-enzyme into H_2O_2 and oxygen, and the other is the divalent reduction of molecular oxygen with enzymes such as D,L-amino acid oxidase and glucose oxidase (Halliwell and Gutterridge 1985). The production of superoxide radical and H_2O_2 has been substantiated in isolated subcellular organelles such as mitochondria, microsomes and chloroplasts.

Euglena gracilis lacks catalase; however *Euglena* contains AsAP that is localized only in the cytosol, but not in other cell compartments based on the subcellular distribution using linear sucrose density gradient centrifugation (Shigeoka et al. 1980a). *Euglena* contains two Fe- and a Mn-superoxide dismutases (SOD) (Kanematsu and Asada 1979). Therefore, *Euglena* cells should contain a H_2O_2-generating system. However, direct evidence on the generation of H_2O_2 in *Euglena* cells remains unknown. This chapter reports on the formation of H_2O_2 in intact chloropasts and mitochondria from *Euglena* cells and demonstrates that H_2O_2 generated in each organelle immediately diffuses into the cytosol.

Materials and Methods

Materials—— Horseradish peroxidase, Grade I-C, RZ ($A_{403\ nm}/A_{275\ nm}$) $\geq$ 3.3, was purchased from Toyobo Co. Ltd. Tokyo. Homovanillic acid was obtained from Sigma. All other chemicals purchased from commercial sources were of the highest purity available.

Organism and culture—— Heterotrophic cultures of *Euglena gracilis* Z were obtained by culturing the unicellular alga at 26°C in Koren-Hutner medium (Koren and Hutner 1967) under illumination (35 $\mu E\ m^{-2}\ s^{-1}$) for 5 days, as described in Chapter II.

Isolation of organelles—— All procedures were carried out at 0-4°C. Partial trypsin digestion of the pellicles followed by mild mechanical disruption of *Euglena* cells and subcellular fractionation by differential centrifugation were conducted according to Shigeoka et al. (1980c). The chloroplast and mitochondrial fractions were further purified by stepwise Percoll density gradient centrifugation as described previously (Isegawa et al. 1984). Photosynthetic CO_2 fixation and ferricyanide reduction were measured in order to elucidate the intact state of isolated chloroplasts (Shigeoka et al. 1980c). The chloroplasts were capable of fixing CO_2 at the rate of 21 μmol of CO_2 mg

chlorophyll^{-1} hr^{-1}: this value corresponded to one-third the rate of whole cells used for isolation of chloroplasts. Ferricyanide reduction was not observed by the chloroplasts, indicating that this compound did not penetrate into the organelles. About 80% of ribulose bisphosphate carboxylase activity, a soluble marker enzyme for chloroplasts, was recovered in chloroplast fractions.

Isolated mitochondria showed satisfactory respiratory control ratio and nearly theoretical P/O ratio with some respiratory substrates. The mitochondria contained about 85% of the total succinate semialdehyde dehydrogenase activity, a mitochondrial soluble marker enzyme. Since these results were in agreement with previous studies of isolated chloroplasts and mitochondria (Shigeoka et al. 1980c, Tokunaga et al. 1976), we concluded that the isolated organelles are physiologically intact and not contaminated by other subcellular fractions.

Determination of H_2O_2 —— The isolated chloroplasts were suspended in 1 m of 25 mM HEPES buffer (pH 7.4) containing 0.33 M mannitol to the final concentration of 200 μg chlorophyll ml^{-1}, bubbled with 100% oxygen for 3 min, and then illuminated at 750 μE m^{-2} s^{-1}. At each time point after illumination, the chloroplast preparation (100 μl) was centrifuged at 3000 × g for 1 min to obtain the chloroplast and the supernatant fractions. The supernatant represents the external chloroplast fraction. Intact chloroplasts were resuspended in the same volume of 25 mM HEPES buffer (pH 7.4) to rupture them osmotically. The lysate was centrifuged at 100000 × g for 20 min. The obtained supernatant represents the internal chloroplast fraction. H_2O_2 in both fractions was measured fluorometrically using homovanillic acid (Tokunaga et al. 1976). Reaction mixture (4 ml) contained 1.25 mM homovanillic acid, 50 μM horseradish peroxidase, 25 mM phosphate buffer (pH 7.5) and 100 μl of internal and external chloroplast fraction. The fluoresence yield was measured at an excitation of 315 nm and an emmision of 425 nm. The concentration of H_2O_2 in internal and external chloroplasts was expressed as nmol mg chlorophyll^{-1}. Isolated mitochondria were resuspended in 100 μl of 25 mM HEPES buffer (pH 7.4) containing 0.25 M sucrose to the final concentration of 2 mg protein ml^{-1}. H_2O_2 generation in mitochondria was determined spectro-photometrically by monitoring the rate of formation of horseradish peroxidase-H_2O_2 compound in shift of the absorption maximum from 402 to 417 nm ($\Delta\varepsilon$mM=50 litre mmol^{-1} cm^{-1}) (Boveris et al. 1972). The reaction mixture (2 ml) contained 50 mM Tris-acetate buffer (pH 7.4), 0.25 M sucrose, 2 μM horseradish peroxidase, 5 mM respiratory substrate and mitochondrial preparation (0.5 mg of protein ml^{-1}). It was preincubated at 27°C for 3 min prior to the addition of each respiratory substrate. The generation rate of H_2O_2 in mitochondria was represented as nmol mg protein^{-1} min^{-1}. Chlorophyll and protein were determined by the methods of MacKinney (1941) and Bradford (1976), respectively.

Results and Discussion

Generation of H_2O_2 in intact mitochondria —— The generation of H_2O_2 via superoxide radical by the respiratory chain in higher plant and animal mitochondria has been established as a physiological event under aerobic conditions (Halliwell and Gutterridge 1985). The generation of superoxide radical was observed in the regions of NADH dehydrogenase and ubiquinone-cytochrome b segments of the respiratory chain (Boveris et al. 1972, Rich and Bonner 1978). In *Euglena* mitochondria, the sequence of electron carriers that mediate the flow of electrons from respiratory substrates to oxygen via cytochrome oxidase is the same as that found in animal and higher plant mitochondria (Buetow 1989). The addition of each respiratory substrate caused H_2O_2 generation in intact mitochondria (Table 5.1). Lactate, the most effective substrate, yielded H_2O_2 at the rate of 9.5 nmol min^{-1} mg $protein^{-1}$. It has been reported that the rate of H_2O_2 generation calculated using the horseradish peroxidase assay is one-third of that using yeast cytochrome *c* peroxidase assay (Boveris 1972), suggesting that the net formation of H_2O_2 in *Euglena* mitochondria is certainly more than the values shown in Table 5.1. Rat liver and pigeon heart mitochondria produced H_2O_2 at rates of 0.5 nmol min^{-1} mg $protein^{-1}$ with succinate (Boveris 1972) and 20 nmol min^{-1} mg $protein^{-1}$ with either succinate-glutamate or malate-glutamate (Boveris and Chance 1973), respectively.

Table 5.1 Generation of H_2O_2 in intact mitochondria.

Substrate (5 mM)	H_2O_2 generated (nmol min^{-1} mg $protein^{-1}$)
Malate	4.00 ± 0.25
Succinate	0.32 ± 0.07
Lactate	9.47 ± 0.59
Glutamate	2.10 ± 0.11
Glycolate	0.32 ± 0.06

Details of the reaction mixture and the assay of H_2O_2 are described in the "Materials and Methods" section.

When each respiratory substrate is omitted from the reaction mixture in the control, H_2O_2 generation is not observed.

Values are means of three assays ± S. D.

Generation of H_2O_2 in intact chloroplasts —— In chloroplasts of higher plants, superoxide radical is produced through the autooxidation of the photoreduced, primary electron acceptor in photosystem I and the photoreduced ferredoxin, even under favorable conditions (Asada and Takahashi 1987). H_2O_2 is formed through the dismutation of

superoxide radical by SOD and the chemical reaction by antioxidants such as L-ascorbate and glutathione (Halliwell and Gutterridge 1985, Asada and Takahashi 1987). *Euglena* chloroplasts contain Fe- and Mn-SOD (Kanematsu and Asada 1979). After bubbling of 100% oxygen for 3 min, the intact chloroplasts were illuminated at 750 μE m^{-2} s^{-1} for 10 min, which resulted in the rapid generation of H_2O_2 (Fig. 5.1). The external chloroplast H_2O_2 increased and leveled off in 7.5 min, while the internal chloroplast H_2O_2 increased slightly, corresponding to one tenth of that of external chloroplast H_2O_2. The initial rate of H_2O_2 generation, which was evaluated from the H_2O_2 level external to the chloroplasts, was 5 μmol h^{-1} mg chlorophyll^{-1} (28.7 nmol min^{-1} mg protein^{-1}), corresponding to 25% of the rate of CO_2 fixation in intact chloroplasts.

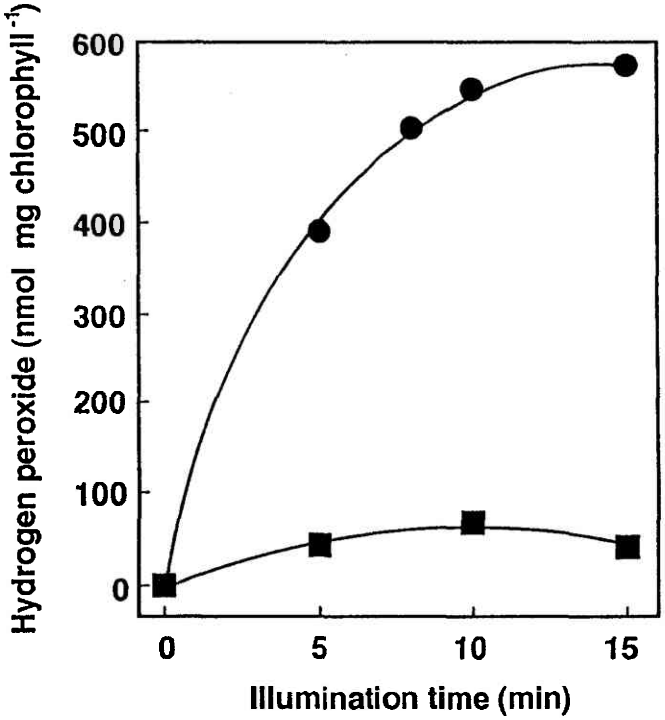

Fig. 5.1 Generation of H_2O_2 in intact chloroplasts. See "Materials and methods" section for details. ■ ,internal chloroplasts; ● , external chloroplasts. Each experimental point represents the mean for four assays (coefficient of variation <5%).

Chloroplasts of higher plants scavenge H_2O_2 by AsAP and also contain MDAsA reductase, DAsA reductase and GSH reductase to constitute the redox cycle of AsA-GSH in combination with AsAP (Asada and Takahashi 1987). H_2O_2 produced in chloroplasts is reduced to water using a photoreductant as the electron donor by a peroxidase reaction. Based on the results reported here and data reported previously (Kanematsu and Asada 1979, Shigeoka et al. 1980b), it is clear that *Euglena* possesses the H_2O_2-generating system and the defense system against toxic active oxygens by two Fe- and one Mn-SOD and the AsA-GSH cycle. The active oxygen-scavenging systems may effectively control

the concentration of oxygen reduction products due to their characteristic subcellular distributions.

The enzymes that constitute the AsA-GSH cycle are exclusively located in the cytosol of *Euglena* cells (Shigeoka et al. 1987a, 1987c). This is also the case in *Acetabularia* (Castillo et al. 1986) and hetrotrophic tissue like soybean root nodules (Dalton et al. 1987). In addition, it has been reported that about 60% of H_2O_2 decomposition in *Euglena* cells was accounted for by the AsA-GSH cycle with a photoreductant as the electron donor linked to photosynthetic electron transport (Miyake et al. 1991). These results raise the question how the AsA-GSH cycle in the cytosol can decompose H_2O_2 generated in each organelle. At each time point after illumination, the H_2O_2 concentration was determined in internal and external chloroplast fractions. The remarkable accumulation of H_2O_2 was only observed in the external chloroplast fraction, but not in the internal chloroplast fraction (Fig. 5.1). The results of H_2O_2 generation in intact mitochondria (Table 5.1) showed that horseradish peroxidase reacted with H_2O_2 produced after the addition of a respiratory substrate and immediately diffused from mitochondria, since mitochondrial membranes were impermeable to the peroxidase. The evidence reported here indicates that H_2O_2 generated in chloroplasts and mitochondria must diffuse immediately from each organelle into the cytosol and then decompose by the AsA-GSH cycle, including AsAP. It has been found that H_2O_2, unlike the superoxide radical, can easily penetrate complex multilayered cell walls and plasma membranes since the pKa of H_2O_2 ($H_2O_2 \leftrightarrow HO_2^- + H^+$) is 11.8 and H_2O_2 exists in a neutral form at physiological pH (Chance et al. 1979, Hayakawa et al. 1984). The production rate of superoxide radical and H_2O_2 was estimated to be about 160 and 80 $\mu M\ s^{-1}$, respectively in chloroplasts of higher plants under normal conditions (Asada and Takahashi 1987). Low concentration of H_2O_2 (10μM) strongly inhibited CO_2 fixation within several seconds (Kaiser 1976). Enzymes such as fructose 1,6-bisphosphatase (FBPase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ribulose-5-phosphate kinase (Ru5P-K) related to the CO_2 fixation cycle are inhibited by H_2O_2, diminishing leaf photosynthesis.

As described in Chapter II, it is an interesting problem why the *Euglena* cells localize AsAP only in the cytsol, but not in the chloroplasts, to scavenge H_2O_2 generated *in vivo*. In this chapter, I demonstrated that H_2O_2 formed in *Euglena* chloroplasts diffuses from this organelle into the cytosol. Recently, Takeda et al. (1995) found that photosynthesis of algal cells including *Euglena* is resistant to H_2O_2 up to 1 mM compared to that of higher plant chloroplasts, which is due to the insusceptibility of the thiol-modulated enzymes such as FBPase, GAPDH, and Ru5P-K to H_2O_2. I conclude that the resistance of photosynthesis to H_2O_2 and the diffusion of H_2O_2 from chloroplasts into the cytosol as well as the H_2O_2-scavenging by AsAP seems to be a protective system against oxidative stress in *Euglena* cells.

Summary

H_2O_2 generation in intact mitochondria and chloroplasts was investigated in *Euglena gracilis*. In mitochondria, the addition of respiratory substrate caused the formation of H_2O_2. Lactate, the most effective substrate, yielded H_2O_2 at the rate of 9.5 nmol min^{-1} mg $protein^{-1}$. Chloroplasts showed a rate of H_2O_2 generation of 5 μmol h^{-1} mg $chlorophyll^{-1}$ under illumination. The results reported here demonstrate that H_2O_2 is generated in mitochondria and chloroplasts and immediately diffuses from each organelle into the cytosol.

CHAPTER VI

Purification and Characterization of Cytosolic Ascorbate Peroxidase from Komatsuna (*Brassica rapa*)

Ascorbate peroxidase (AsAP) has been found to occur as at least three distinct isozymes: the cytosolic isoform, and two chloroplastic isoforms, one in the stroma and the other associated with the thylakoid membranes (Asada 1994). The physiological role of the chloroplastic AsAP is particularly well documented (Asada 1994). Thiol-modulated enzymes such as fructose 1,6-bisphosphatase and glyceraldehyde-3-phosphate dehydrogenase in photosynthetic carbon reduction (PCR) cycle are readily inactivated if the AsAPs do not serve to rid cells of excess H_2O_2 (Kaiser 1976, Asada 1994). Mittler and Zilinskas (1991) reported that cytosolic AsAP is indeed a major component of enzymatic oxyradical scavenging system in pea leaves. Schantz et al. (1995) pointed out that different expression of isoforms occurs in the cytosol as well as in the plastidal compartment in bell pepper. A novel AsAP isozyme has been found to be localized on membranes of microbodies in pumpkin and its active site was exposed to the cytosol (Yamaguchi et al. 1995). Furthermore, it has been known that non-photosynthetic tissues such as root nodules of legumes (Dalton et al. 1987) and potato tubers (Elia et al. 1992) contain only cytosolic AsAP. Although these facts suggest that AsAP is a multigene family and cytosolic AsAP plays a major role in scavenging H_2O_2 in a case of some stress and development conditions, the physiological properties of cytosolic AsAP remain still unclear. Thus, in photosynthetic organism, it is necessary to elucidate a detailed physiological functions of cytosolic AsAP.

In order to understand a clue to the physiological roles of cytosolic isozyme, this chapter reports the ratios of activities of AsAP isozymes and purification and some properties of cytosolic AsAP in komatsuna (*Brassica Rapa*) leaves.

Materials and Methods

Plant material —— Freshly harvested komatsuna (*Brassica rapa*) were purchased at the local market and stored at 4°C in the dark until used. Stems and petioles were removed before use.

Enzyme assays —— AsAP was measured by the decrease in the absorbance at 285 nm due to ascorbate oxidation as described in Chapter II. The reaction mixtures contained 50 mM potassium phosphate (pH 6.3), 0.4 mM AsA, and 0.1 mM H_2O_2. Oxidation of alternate electron donors was measured in the same assay mixture as that

used for ascorbate, but ascorbate was replaced by 20 mM pyrogallol (430 nm, 2.47 mM^{-1} cm^{-1}), 20 mM dianisidine (460 nm, 11.3 mM^{-1} cm^{-1}).

Separate assay for ascorbate peroxidase isozymes—— Separate assay for AsAP isozymes was done by the recommended procedure of Amako et al. (1994). The principle of this method exploit the different sensitivities of the isozymes to inactivation in AsA-depleted medium. The crude extract prepared according to the description below. The extract (1 ml) was passed through a gel-filtration column of Sephadex G-25 (10 × 500 mm), which had been equilibrated with the 10 mM potassium phosphate buffer (pH 7.0) containing 20% sorbitol, 1 mM EDTA and 100 μM AsA, and the void volume fraction (1ml) was used as the assay sample.

Cytosolic ascorbate peroxidase purification—— All steps in the purification were carried out at 4°C. Komatsuna leaves were homogenized in 300 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 20% (w/v) sorbitol, 1 mM AsA, 1% polyvinylpyrolydone by mortar and pestle. The homogenate was filtered through eight layers cloth and then centrifuged at 8000 × g for 20 min to remove cell debris. The supernatant will be referred to in this report as the crude extracts. Crude extracts loaded onto a DEAE-Sephacel column (2.3 × 27 cm) equilibrated in 10 mM phosphate buffer (pH 7.0) containing 20% sorbitol, 1 mM AsA, and 1 mM EDTA. The column was eluted with 300 ml of a linear gradient of 0-300 mM NaCl. Active fractions were collected and subjected to ammonium sulfate precipitation at 30% saturation. The precipitate was removed by centrifugation at 15000 × g for 20 min and the supernatant was loaded onto a butyl-Toyopearl column (1.2 × 16 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 20% sorbitol, 1 mM AsA, 1 mM EDTA, and ammonium sulfate (20% saturation). The column was washed with 200 ml of the equilibration buffer, and the enzyme activity was eluted with a descending gradient of ammonium sulfate from 30 to 0% saturation made in 300 ml of the same buffer. Two peaks designated P-I and P-II, were eluted by ammonium sulfate at approximately 20% and 15% concentration, respectively. The active fraction of P-II, which corresponded with cytosolic AsAP, was pooled and concentrated by ultrafiltration (through an Amicon PM-10 membrane) using centricon-10 concentrator (Amicon). The preparation of concentrated fraction was applied to a gel filtration column (0.75 × 60 cm) of TSK-gel G3000 SW (TOHSO, Japan), equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 20% sorbitol, 1 mM EDTA, 1 mM AsA, and 0.1 M NaCl, and eluted with the same buffer. The purified enzyme was stored at -20°C prior to use.

SDS-PAGE—— SDS-PAGE on slab gel was performed by the method of Laemmli with 5% acrylamide for the stacking gel and 15% acrylamide for the separating gel. Samples were denatured by boiling for 3 min in 0.1% (w/v) SDS in the presence of 5% (v/v) 2-mercaptoethanol.

Protein sequencing —— Purified AsAP was analyzed by automated Edoman degradation on a protein sequencer (Applied Biosystems model 470A) equipped with a phenylthiohydantoin analyzer (model 120A) as described in Chapter II.

Immunoblotting —— Proteins were separated using SDS-PAGE and blotted onto an Immobilon-P transfer membrane (PVDF, pore size 0.45 μm, No. IPVH 304FO, Millipore, Bedford, MA) using a semidry electroblotting system according to the manufacturer's instructions (Bio-Rad). Immunoblotting was performed according to the method of Towbin et al. (1979). *Euglena* monoclonal antibodies were prepared as described in Chapter II.

Protein assay —— Protein was measured according to Lowry et al.(1951) as described in Chapter II using bovine serum albumin as standard.

Results and Discussion

The ratio of activities of ascorbate peroxidase isozymes in komatsuna leaves —— In order to examine the ratios of activities of chloroplastic and cytosolic AsAP in komatsuna leaves, I exploited separate assay of AsAP according to Amako et al. (1994).

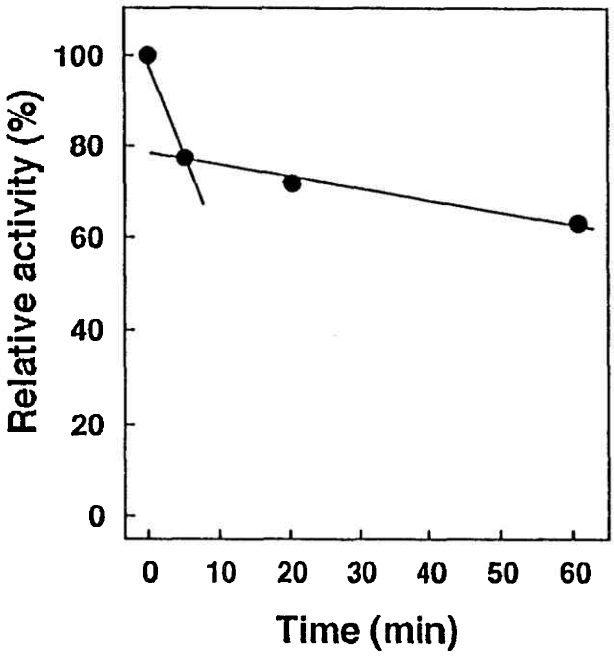

Fig. 6.1. The time course of inactivation of AsAP in extracts from komatsuna leaves. The crude extract from komatsuna leaves was prepared in 10 mM potassium phosphate buffer (pH 7.0) containing 20% (w/v) sorbitol and 1 mM AsA as described in " Materials and Methods". The extract (1 ml) was passed through a gel-filtration column of Sephadex G-25 (10 × 500 mm), which had been equilibrated with the 10 mM potassium phosphate buffer (pH 7.0) containing 20% sorbitol, 1 mM EDTA and 100 μM AsA, and the void volume fraction (1ml) was used as the assay sample. The incubation was started to 20-fold dilute the extract in the 10 mM potassium phosphate buffer (pH 7.0) containing 20% sorbitol, 1 mM EDTA. The inactivation in AsA-depleted medium was stopped by the addition of AsA to final concentration of 1 mM in each time periods. AsAP activity was assayed according to "Materials and Methods".

The extracts from komatsuna leaves showed two-phase kinetics of inactivation of AsAP (Fig. 6.1). It has been known that chloroplastic AsAP and cytosolic AsAP are 50% inactivated in the absence of AsA by within approximately 15s and 40 min, respectively (Chen and Asada 1989). The measurement of their inactivities allowed us to estimate the activities of chloroplastic and cytosolic AsAP isozyme ratios of komatsuna leaves. Table 6.1 shows comparison of the ratios of chloroplastic and cytosolic AsAP activities from various higher plants including komatsuna leaves. In komatsuna leaves, chloroplastic AsAP activity was a low ratio (approximately 20%) compared with cytosolic AsAP activity. From these results, it is important to note that higher plants can be classified into two types with respect to the ratio of chloroplastic AsAP to cytosolic AsAP activities : one type is a high ratio of cytosolic enzyme activity like komatsuna and maize leaves, and the other type is a high ratio of chloroplastic AsAP activity as tea and spinach leaves. Chapter II showed that, in *Euglena* cells, AsAP occurs only in the cytosol but not in the chloroplasts.

Table 6.1. Separate assay of chloroplastic (sAsAP) and cytosolic (cAsAP) isozymes of AsAP in leaves.

	AsAP activity ratios	
	cAsAP (%)	sAsAP (%)
Komatsuna	76	24
Cucumber*	70	30
Rice*	65	35
Maize*	80	20
Spinach*	35	65
Tea*	25	75
Euglena gracilis	100	0

The activities of AsAP isozymes were determined by the separate assays described in "Materials and Methods". The asterisks are from reference of Amako and Asada (1994).

Purification of cytosolic ascorbate peroxidase from komatsuna leaves—— Chloroplastic and cytosolic isozymes of AsAP has been found in tea and spinach leaves, and their properties were characterized (Chen and Asada 1989, Tanaka et al. 1991). The chloroplastic isozyme is specific to AsA. In contrast, the cytosolic isozyme can oxidize pyrogallol at an appreciable rate (Asada 1994). One of the characteristic properties of AsAP isozymes is inactivation in the AsA-depleted medium. This is especially the case for chloroplastic AsAP, whose half-inactivation time is only 15s. Cytosolic AsAP is more stable than the chloroplastic type (Asada 1994). In the first chromatographic step a single peak of AsAP activity was obtained with the yield of 84.0%. At the purification step using butyl-Toyopearl column chromatography, the AsAP activity was separated

into the major active peak and minor one designated P-II and P-I, respectively (Fig. 6.2). P-I and P-II were eluted by ammonium sulfate at approximately 20% and 15% concentration, respectively. The both peaks showed sharp and single peak and no detectable any other peaks were observed. In a AsA-depleted medium, P-I was particularly labile as well as spinach and tea chloroplastic isozymes, while, P-II was more stable than P-I as described below. P-I is specific to AsA as the electron donor and P-II can utilize not only AsA but also pyrogallol. Thus, I discriminated that P-I and P-II were chloroplastic and cytosolic AsAP, respectively. The P-II was further purified as described in "Materials and Methods" section. A typical purification steps of komatsuna cytosolic AsAP are summarized in Table 6.2. The purification of komatsuna cytosolic AsAP resulted in a 100-fold increase in specific activity and a final yield of 8.0%. The purified enzyme had a specific activity of 56.0 μmol min^{-1} mg $protein^{-1}$. The purification was repeated several times with similar results. These results indicate that as regards cytosolic AsAP, komatsuna leaves contain only one type isozyme.

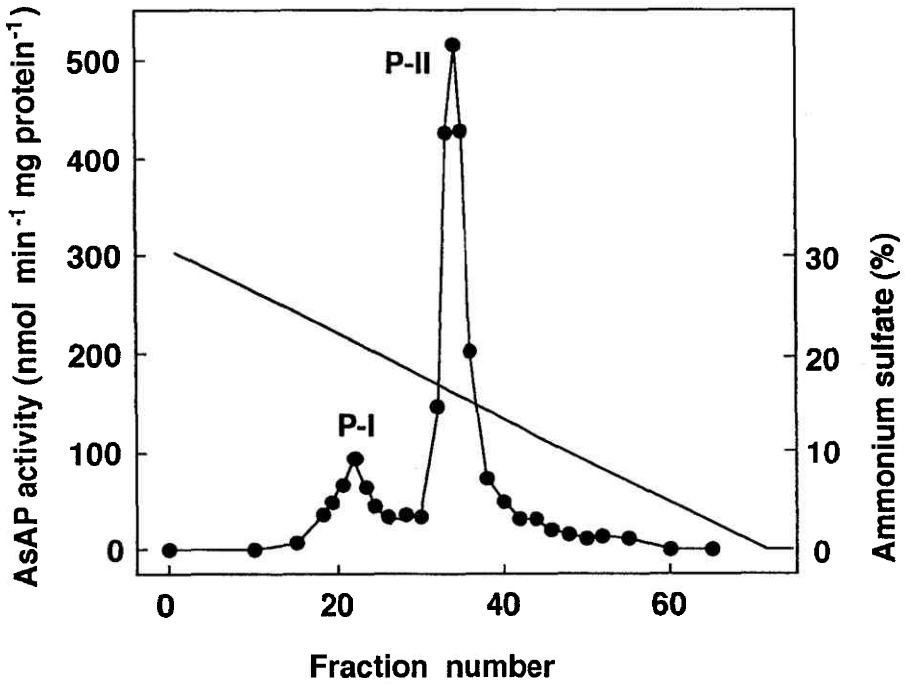

Fig. 6.2. Elution pattern of AsAP on butyl-Toyopearl column chromatography. Detailed procedures were presented in "Materials and Methods".

The N-terminal sequence of komatsuna cytosolic AsAP could not be determined by Edoman degradation on the protein sequencer, indicating that the N-terminus residue of this enzyme was blocked. This is the first case as far as I know. N-Terminal sequences of cytosolic AsAPs from various higher plants are determined and there is a high degree of homology between different sequences of cytosolic AsAPs (Mittler and Zilinskas 1991b, Tanaka et al. 1991, Kubo et al. 1992)

Table 6.2. Purification of AsAP from komatsuna (*Brassica rapa*) leaves.

Step	Total protein (mg)	Total activity (μmol min^{-1})	Specific acitivity (μmol min^{-1} mg protein^{-1})	Fold	Yield (%)
Crude extract	497.0	279.7	0.56	1.0	100
Ultra-centrifugation	493.2	272.6	0.55	1.0	97.5
DEAE-Sephacel	30.9	234.9	9.29	16.6	84.0
30% $(NH_4)_2SO_4$	21.6	221.8	10.27	18.3	79.3
Butyl-Toyopearl	1.9	83.9	14.90	26.6	30.0
TSK G3000SW	0.4	22.4	56.00	100	8.0

Details of the purification are described in "Materials and Methods".

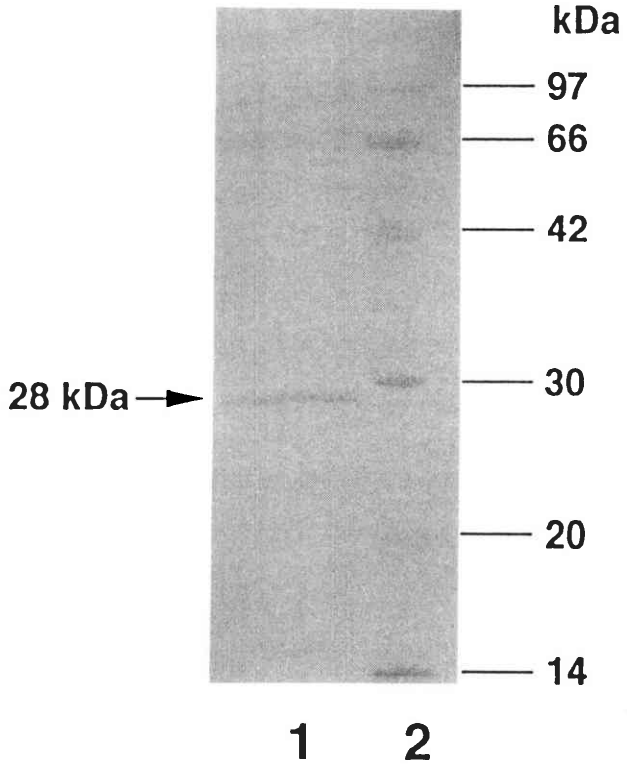

Fig. 6.3. SDS-PAGE analysis of the purified cytosolic AsAP from komatsuna leaves. Polyacrylamide gel concentration was 12.5%. 5 μg of purified enzyme (lane 1) and molecular mass protein standards (lane 2) were subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250. The arrow indicates the AsAP.

The glycoprotein nature of the purified komatsuna cytosolic AsAP was examined. The purified enzyme was separated by SDS-PAGE and electrophoretically blotted onto PVDF-membranes and then glycochains detected using G.P.sensor (Honen Corp., Japan). The result showed the komatsuna AsAP was not glycosylated by α-D-

mannopyranosyl and α-D-glucopyrosyl residues as previously report (Chen and Asada 1989) (data not shown).

Coomassie-stained SDS-PAGE of the final preparation showed a single band corresponding to molecular mass of 28000 Da (Fig. 6.3). Gel filtration chromatography with a TSK-gel G3000 SW column (TOHSO, Japan) revealed that the isolated komatsuna cytosolic AsAP had a native molecular mass of 28000 ± 1000 Da (Fig. 6.4).

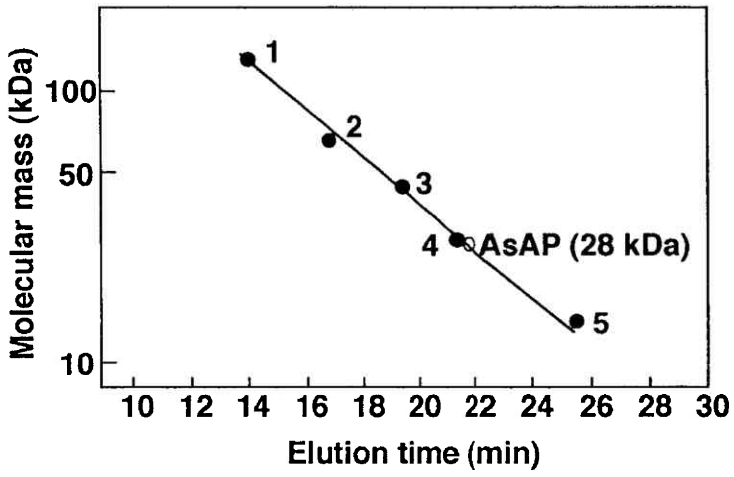

Fig. 6.4. Molecular mass determination of komatsuna cytosolic AsAP by HPLC on TSK-gel G3000 SW. The detailed procedures were described in "Materials and Methods". The purified enzyme (3 μg) was injected into HPLC column. The column was calibrated with the molecular mass standard proteins as following: 1, BSA (dimer; 132 kDa); 2, BSA (monomer; 66 kDa); 3, chicken egg albumin (45 kDa); 4, carbonic anhydrase (29 kDa); 5, α-lactalbumin (14.2 kDa).

These results indicate komatsuna cytosolic AsAP exists a monomeric form in its native state. In pea leaves, cytosolic AsAP occurs as a homodimer of two subunits, which did not associate via disulfide bonds, with molecular mass of approximately 29500 Da (Mittler and Zilinskas 1991a). Previous study with purified cytosolic AsAP from potato tubers indicated native molecular mass of 32000 Da and had a subunit molecular mass of 30000 Da (Elia et al. 1992). In contrast with cytosolic AsAP, the spinach thylakoid-bound and tea stromal isozymes were found a monomeric form with molecular mass of 40000 Da and 34000 Da, respectively (Miyake et al. 1993, Chen and Asada 1989). These results indicate that cytosolic AsAPs in higher plants exist as two forms in their native states which are dimeric and monomeric. Chapter II showed that in an AsA-depleted medium *Euglena* cytosolic AsAP has an exceptionally high molecular mass and is a monomeric form with a molecular mass of 58000 Da.

Characterization of cytosolic ascorbate peroxidase from komatsuna leaves——The optimum pH of the purified cytosolic AsAP was 6.5 and the optimum temperature was 39°C (Fig. 6.5). Previous reports with respect to cytosolic and chloroplastic AsAP isozymes from various sources indicate optimum pH ranges of 6.0-8.0 (Mittler and

Zilinskas 1991a, Elia et al. 1992, Chen and Asada 1989, Miyake et al. 1993). The enzyme retained full activity up to 35°C between pH 6.5 and 7.5 and lost activity completely at 55°C (Fig. 6.5).

AsAP utilizes AsA as the most effective electron donor (Asada 1992). Unlike chloroplastic AsAP, the cytosolic isozyme can utilize alternate electron donors, such as pyrogallol and guaiacol (Asada 1992). Utilization of alternate electron donors is demonstrated in Table 6.3. In agreement with previous studies (Chen and Asada 1989, Mittler and Zilinskas 1991a), komatsuna cytosolic AsAP was able to catalyze the oxidation of pyrogallol at a 2.5-fold higher rate than that of AsA.

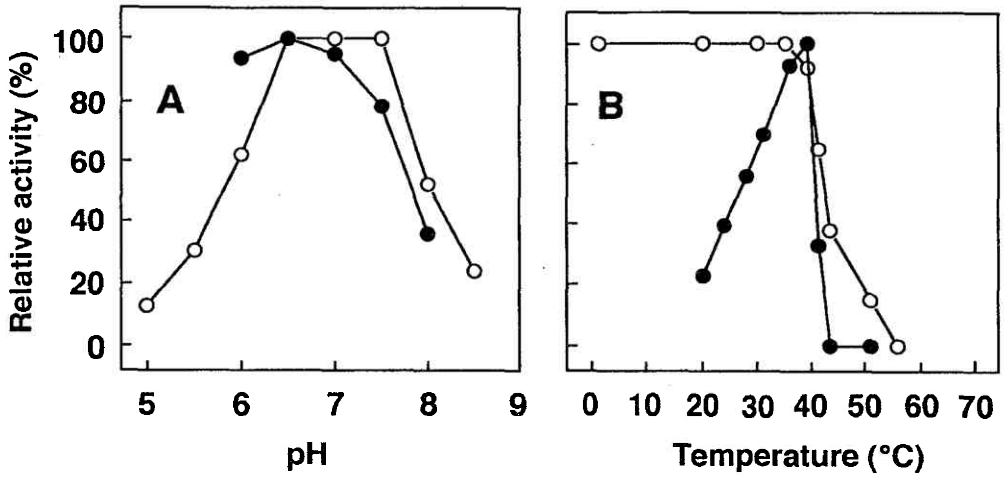

Fig. 6.5. Optimum pH and temperature, and pH and thermal stability of komatsuna cytosolic AsAP. A shows the optimum pH and pH stability. B shows the optimum temperature and thermal stability. The optimum pH was determined by using 50 mM Tricine buffer (pH 7.5-8.5), 50 mM potassium phosphate buffer (pH 6.0-8.0), 50 mM HEPES buffer (pH 7.0-8.0) and 50 mM Tris-acetate buffer (pH 5.0-9.0). The reaction mixture (2 ml) contained 100 mM of the phosphate buffer described above and "Materials and Methods". The pH in the reaction mixture was measured at 37 °C on pH meter (HORIBA) after the determination of the activity. The pH stability was determined with the enzyme pretreated at various pH value for 15 min at 40 °C. Relative activity was expressed in percent against the activity of pH 6.5. The specific activity at pH 6.5 was 60.0 μmol min^{-1} mg $protein^{-1}$. The optimum temperature was determined by incubation between 4 and 55 °C with 50 mM phosphate buffer (pH 7.0). The thermal stability was measured with the enzyme preincubated at various temperatures for 15 min at pH 7.0. Relative activity was expressed in percent against the activity of pH 7.0. The specific activity at pH 7.0 was 56.0 μmol min^{-1} mg $protein^{-1}$. For details see "Materials and Methods".

The apparent *K*m values for AsA and H_2O_2 were determined to be 402 μM and 24 μM, respectively, from the double reciprocal plots of substrate concentrations and reaction rates (Fig. 6.6). These values were of the same order of magnitude as those reported for cytosolic and chloroplastic AsAPs from other sources; the *K*m values for AsA and H_2O_2 are in the ranges 220-480 μM and 20-80 μM in photosynthetic organisms, respectively (Shigeoka et al. 1980a, Mittler and Zilinskas 1991a, Chen and Asada 1989).

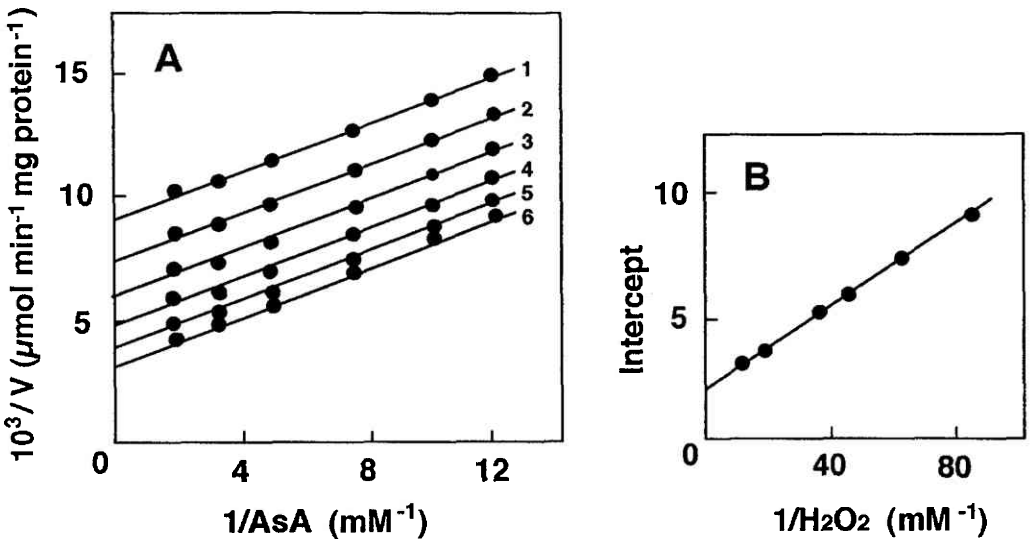

Fig. 6.6. Double reciprocal plots of komatsuna cytosolic AsAP activity in various concentrations of both AsA and H2O2. A; Double reciprocal plots of initial velocity against variable AsA concentrations at several fixed H2O2 concentrations. H2O2 were 0.0110 (line 1), 0.0168 (line 2), 0.0221 (line 3), 0.0294 (line 4), 0.0442 (line 5), and 0.0735 (line 6) mM. B; Replots of intercepts against reciprocal H2O2.

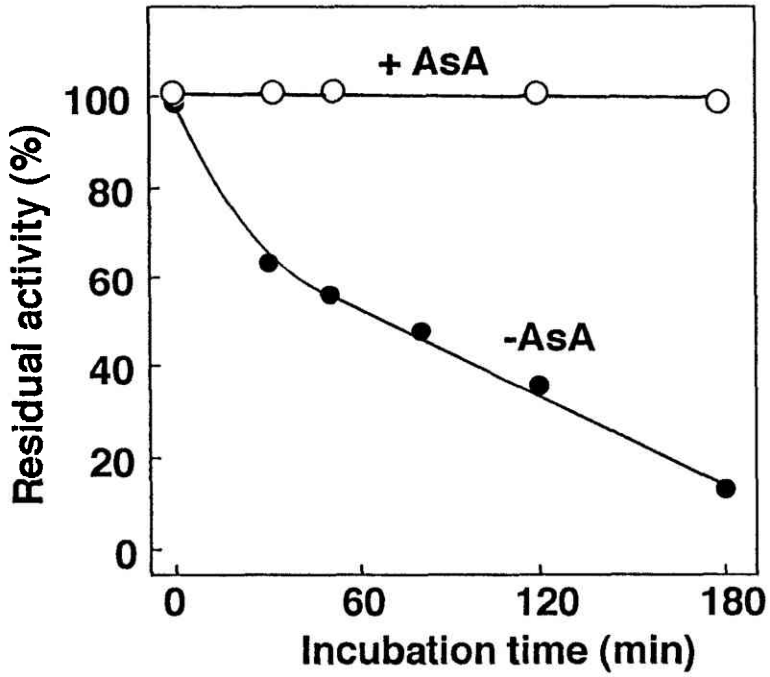

Fig. 6.7. The inactivation of purified cytosolic AsAP from komatsuna leaves by depletion of AsA. Purified AsAP stored in a medium containing 10 mM potassium phosphate (pH 7.0), 100 μM AsA and 20% sorbitol was diluted with 10 mM potassium phosphate (pH 7.0) at the final AsA concentration of below 10 μM (-AsA). After the dilution, 1 mM AsA was added at the indicated times and AsAP activity was assayed according to "Materials and Methods". Where indicated (+AsA), the dilution was done with the buffer containing 1 mM AsA.

One of the characteristic properties of higher plant AsAPs is inactivation in the absence of AsA as described above. The half-inactivation time of the purified komatsuna cytosolic AsAP was approximately 80 min (Fig. 6.7). This is also the case of already known cytosolic AsAP (Chen and Asada 1989).

The purified enzyme was strongly inhibited 87.1% and 82.0% by 0.5 mM azide and 10 μM cyanide, respectively (Table 6.3), indicating that komatsuna cytosolic AsAP is a typical hemeprotein as well as other AsAPs.

Table 6.3. Summary of some properties of cytosolic AsAP from komatsuna (*Brassica rapa*) leaves.

Moleculer mass (Da)		28000
Optimum pH (stability)		6.5 (6.5 - 7.5)
Optimum Temp. (stability)		39°C (35°C >)
Donor specificity (%)		
AsA		100
GSH		0
Cyt *c*		0
NAD(P)H		0
Pyrogallol		250
Guaiacol		0
o-Dianisidine		6.5
*K*m value (μM)		
AsA		402
H_2O_2		24
Effect of inhibitors (%)		
NaN_3	0.5 mM	87.1
	1.0 mM	100
KCN	10.0 μM	82
	50.0 μM	100
Carbohydrate		none

Immunoblot analysis of komatsuna ascorbate peroxidase using Euglena *monoclonal antibody*—— Immunoblot analysis was performed using *Euglena* mAb (EAP1) which cross-reacted with both chloroplastic and cytosolic AsAPs from higher plants (Chapter II). The purified komatsuna cytosolic AsAP cross-reacted with the *Euglena* mAb at a position corresponding to a molecular mass of 28000 Da (Fig. 6.8). This result showed that komatsuna cytosolic AsAP and already known cytosolic AsAPs share common epitopes. The immunoblot analysis with crude extract from komatsuna leaves showed only one protein band corresponding to a purified enzyme, supporting komatsuna leaves contain a only one type of cytosolic AsAP.

In conclusion, komatsuna leaves exhibited a high ratio of cytosolic AsAP to chloroplastic AsAP. The enzymatic characterization of komatsuna cytosolic AsAP agreed very closely with those of already known cytosolic AsAPs.

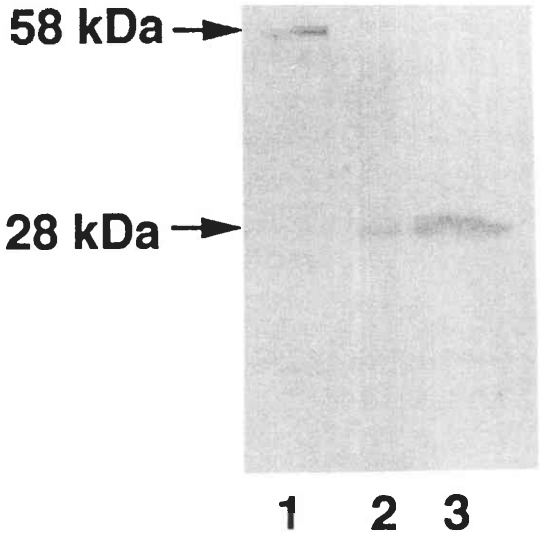

Fig. 6.8. Immunoblotting of purified cytosolic AsAP from komatsuna with the monoclonal antibody against *Euglena* AsAP after SDS-PAGE. Detailed procedures were presented in "Materials and Methods". Lane 1, purified *Euglena* AsAP (5 μg); Lane 2, crude extract from komatsuna leaves (50 μg); Lane 3, purified komatsuna cytosolic AsAP (3 μg).

In plant cells, it is well known that production of active oxygen species including H_2O_2 increases as the results from some environmental stresses. Accordingly, higher plant cells response to changing their scavenging systems to minimize damage to the metabolic apparatus (Gillham and Dodge 1987, Jahnke et al. 1991, Mittler and Zilinskas 1994). In komatsuna leaves, it is necessary to further investigate the physiological function of cytosolic AsAP including its response to environmental stresses.

Summary

In komatsuna (*Brassica rapa*) leaves, the activity of chloroplastic ascorbate peroxidase was a low ratio (approximately 20%) compared with that of cytosolic ascorbate peroxidase judged from the separation assay. The cytosolic ascorbate peroxidase was purified to homogeneity from komatsuna leaves and examined some properties. The purified enzyme was a monomer with a molecular mass of 28 kDa according to gel filtration and SDS-PAGE. The activity was maximal at pH 7.0 and 38°C, and stable between pH 7.0 and pH 8.0 until 35°C. The inhibitions of the enzyme by cyanide and azide showed that it is a hemoprotein. This enzyme was not glycoprotein. When the enzyme presented in ascorbate-depleted medium, the half inactivation time was approximately 80 min. These results indicated that this enzyme was the typical cytosolic ascorbate peroxidase.

CHAPTER VII

Cloning and Expression of cDNA Encoding a New Type of Ascorbate Peroxidase from Spinach

Plant-type peroxidases have been grouped in three classes, which are distantly related structurally. Class I is formed by prokaryotic lineages such as yeast cytochrome *c* peroxidase (CCP) and ascorbate peroxidase (AsAP). Secretory fungal peroxidases (manganese and lignin peroxidases) belong to class II, while the classical plant peroxidases such as GP belong to class III (Welinder 1992).

The organization of the cDNA clones coding the cytosolic AsAP has been reported in pea (Mittler and Zilinskas 1991), *Arabidopsis* (Kubo et al. 1992), soybean (Chatfield and Dalton 1993), maize (Breusegem et al. 1995), and spinach (Webb and Allen 1995). In Chapter II, monoclonal antibodies (mAb; EAP1 and EAP2) raised against *Euglena* AsAP cross-reacted with cytosolic and chloroplastic AsAP in spinach leaves. This chapter reports the cloning and expression in *E. coli* of two cDNA clones encoding a new type of AsAP and already known cytosolic AsAP from spinach leaves using the mAb as a probe.

Materials and Methods

Materials —— A spinach (*Spinacia oleracea*) cDNA library was purchased from Stratagene. Goat anti-mouse antibodies were obtained from Boehringer Mannheim. GP from spinach leaves was generously supplied by Dr. Kozi Asada (Kyoto University). All other chemicals were reagent grade and used without further purification.

Screening of the cDNA library —— A spinach cDNA library in λZAP was screened by mAbs raised against *Euglena* AsAP. The purification of *Euglena* AsAP and mAbs preparation are described in Chapter II. Recombinant pBluescript SK- phagemids were rescued from positive bacteriophage clones by *in vivo* excision by the procedure provided by the manufacturer. The nucleotides of the isolated cDNA clone were sequenced by the dideoxy chain primer method modified for double stranded plasmid DNA.

Preparation of recombinant protein —— *E. coli* cells were grown with shaking at 37°C to an OD_{600} of 0.6-0.7 in Luria-Bertani medium containing 100 mg/ml ampicillin. Expression was then induced by adding IPTG to 1.0 mM and shaking for 3 h more at 37°C. Cells were harvested by centrifugation at 500 × g for 20 min. After resuspension of the pellet in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM AsA and 1 mM EDTA, the cells were disrupted by sonication (10 kHz) for 3 min. The supernatant

obtained by centrifugation at 10000 x g for 15 min was used as the crude recombinant enzyme.

Assay of peroxidase activity—— The activity of AsAP was determined spectrophotometrically as described in Chapter II. Oxidation of alternate electron donors was measured in the same assay mixture as that used for AsAP, but AsA was replaced by 20 mM pyrogallol (430 nm, 2.47 mM^{-1} cm^{-1}) or 20 mM guaiacol (460 nm, 11.3 mM^{-1} cm^{-1}).

Northern blot analysis—— Total RNA was extracted from leaves and roots of spinach as described in reference (Chomczynski and Sacchi 1987). Poly(A)$^+$-RNA was prepared by using PolyATtract mRNA Isolation Systems (Promega). Total RNA (20 μg) and Poly(A)$^+$-RNA (2 μg) were electrophoresed in a formaldehyde/1.2% agarose gel, blotted on a nylon membrane filter, and hybridized to a ^{32}P-labeled probe. For Northern blotting, SAP1 and SAP4 were used as each probes. The membranes were washed in 0.2 x standard saline citrate/0.1% SDS at 60°C for 20 min.

Results and Discussion

Isolation and characterization of cDNA encoding ascorbate peroxidase—— The spinach cDNA library was screened with the monoclonal antibodies raised against *Euglena* AsAP (Chapter II). Two positive clones designated SAP1 and SAP4, containing inserts of 1.2-kb and 1.1-kb, respectively, were isolated and completely sequenced (Fig. 7.1, 7.2).

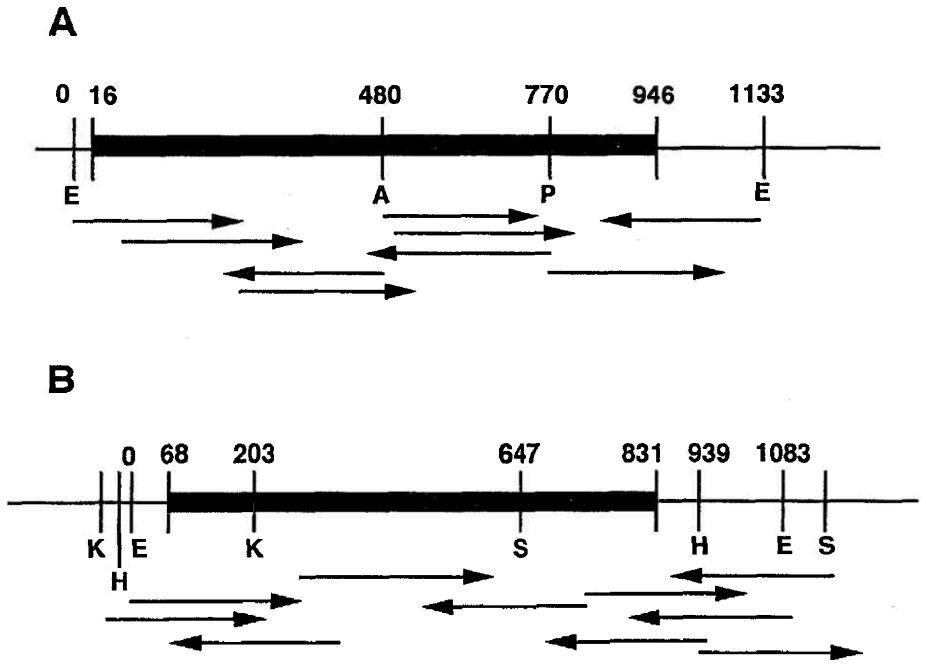

Fig. 7.1. Partial restriction map and sequencing strategy for SAP1 (A) and SAP4 (B). Restriction enzymes are shown as follows: A, *Apa*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sac*I. The coding sites are boxed. Arrows indicate the direction and extent of sequencing.

The nucleotide sequence of SAP1 consisted of 927 bp in the coding region. The ORF coded for a protein of 309 amino acids. The calculated molecular mass of the cloned sequence in SAP1 was 34471 Da. On the other hand, the 1.1-kb insert of SAP4 contained an ORF coding for a protein of 250 amino acid residues, which was calculated to be 27560 Da. Neither cloned cDNA had a transit peptide. The nucleotide sequences and the primary structure of the deduced SAP4 protein agreed with the spinach cytosolic AsAP (Webb and Allen 1995), and the deduced amino acid sequences of the N terminal region was homologous with the sequenced N terminus of spinach cytosolic AsAP reported by Tanaka et al. (1991). In contrast, the deduced N terminus of SAP1 was little homologous with cytosolic AsAPs including SAP4 and the classical plant peroxidases (Mittler and Zilinskas 1993, Koshiba 1993, Welinder 1976).

A

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NGATCAAGTTCCAAAATGGGAAGAGTACCAATTGTGAATGAAAACTATCGGAGGGTAATT     60
                M  G  R  V  P  I  V  N  E  N  Y  R  R  V  I      15
GAGGCAGCTCGTCGAGACCTCCACCGTTCTCTCGTCCAGGACAACAACAACTCTGCTCCT    120
E  A  A  R  R  D  L  H  R  S  L  V  Q  D  N  N  N  S  A  P      35
ATCCTCCTCCGCCTCTCATTTCACGATGCAGTGGACTATGATGCAGCAACAAAACGAGGT    180
I  L  L  R  L  S  F  H  D  A  V  D  Y  D  A  A  T  K  R  G      55
GGTGCAAATGGTTCAGTTAGGTTAGCTCAGGAGTTGAACCGCACCCCAAACAAGGGCATA    240
G  A  N  G  S  V  R  L  A  Q  E  L  N  R  T  P  N  K  G  I      75
GAAACTGCTGTTAGATTTTGTGAGCCGATCAAGCGTAGACACCCTGATATTACATATGCT    300
E  T  A  V  R  F  C  E  P  I  K  R  R  H  P  D  I  T  Y  A      95
GACCTTTATCAGCTAGCTGGAATTGTTGCAGTAGAAGTAACCGGAGGTCCTGCTATTGAT    360
D  L  Y  Q  L  A  G  I  V  A  V  E  V  T  G  G  P  A  I  D     115
GCTGATGTTGCAGACCAAGACAATATCCCAAACCCTAGAAGAGGAGCGGATCATCTGAGA    420
A  D  V  A  D  Q  D  N  I  P  N  P  R  R  G  A  D  H  L  R     135
ACCGTATTTTATCGAATGGGCCTCAATGACAAGGATATCGTTGTACTTTCTGGGGCCCAC    480
T  V  F  Y  R  M  G  L  N  D  K  D  I  V  V  L  S  G  A  H     155
GCACTGGGTGGAGCACACAAGGACAGATCAGGGTTTGACGGCGATTTCACTCGAAACCCT    540
A  L  G  G  A  H  K  D  R  S  G  F  D  G  D  F  T  R  N  P     175
TTGACGTTTGATAACTCCTACTTTGTAGAGCTACTGAGGGGTGATACTCCAGGGTTGGTG    600
L  T  F  D  N  S  Y  F  V  E  L  L  R  G  D  T  P  G  L  V     195
AAATTTCCAACAGATAAGGCTCTCTTAACTGACCCTCGTTTCCGTCCCTTTGTTGATCTC    660
K  F  P  T  D  K  A  L  L  T  D  P  R  F  R  P  F  V  D  L     215
TACGCTAGGGATCAAAGAGCATTCTTCAGAGATTATGCAGAATCACACAAGAAGATGTCC    720
Y  A  R  D  Q  R  A  F  F  R  D  Y  A  E  S  H  K  K  M  S     235
CTGCTAGGGCTTAATCATCCGGAATCCAACCTATATGAAAGTAACAGCTGCAGCACGAGG    780
L  L  G  L  N  H  P  E  S  N  L  Y  E  S  N  S  C  S  T  R     255
TTGAGTGTGGTGCTCAATCCTACGTTGTCCAAAACGGAGGCTGTCCAATGCAATACGGAT    840
L  S  V  V  L  N  P  T  L  S  K  T  E  A  V  Q  C  N  T  D     275
ATGCTCGACCCTATGCAGTTGGAAATGGTTGCTGCCCAGGCGGCTACTGATACCTATAAT    900
M  L  D  P  M  Q  L  E  M  V  A  A  Q  A  A  T  D  T  Y  N     295
ATGCCCATATATACGGCTGTTAACTGTAACAGTCTACGTGACTGAGTGAGTACGTGATGG    960
M  P  I  Y  T  A  V  N  C  N  S  L  R  D  *                    309
GAATTAATATCTTTGAGTTCATGTGATGGCCTCAAGAATAATTAAGTTGTAACGTTTTAC   1020
TTTGTTATATATACTCGTTTATTATGTAATTAAAGTTGTAGTAGTAACGGGGAAACTGTA   1080
CGTGACAGGAACTTGCTTGGAGAAAATCAGCCTTCCTGCCCTCCCTGCATGCT          1133
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B

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GCTCACCACACTCATCTTCCGCTTTTTAGGGTTTTTCATTCATTTGATTCTTCATCAGAC    60
TTTAAGCCATGGGAAAGAGCTACCCAACTGTCAGTGAGAACTACCAGAAATCTATTGAAA   120
        M  G  K  S  Y  P  T  V  S  E  N  Y  Q  K  S  I  E  K     18
AGGCCCGGAGAAAGCTCAGGGGTTTGATCGCAGAGAAGCAATGTGCTCCTCTTATGCTTC   180
  A  R  R  K  L  R  G  L  I  A  E  K  Q  C  A  P  L  M  L  R    38
GTCTTGCATGGCACTCTGCTGGTACCTTTGATTGTACTTCAAAAACTGGAGGTCCCTTTG   240
  L  A  W  H  S  A  G  T  F  D  C  T  S  K  T  G  G  P  F  G    58
GTACAATGAAGCACCAGGCAGAGCTGGCTCATGGGGCTAACAATGGGCTTGTTATTGCTG   300
  T  M  K  H  Q  A  E  L  A  H  G  A  N  N  G  L  V  I  A  V    78
TTAGGCTGTTGGAACCCATCAAGGAACAATTCCCCGAAATTACTTATGCTGACTTTTACC   360
  R  L  L  E  P  I  K  E  Q  F  P  E  I  T  Y  A  D  F  Y  Q    98
AGCTGGCTGAGTTTGTGGCCGTTGAAGTTACTGGAGGACCTGAAGTTCCCTTCCACCCAG   420
  L  A  E  F  V  A  V  E  V  T  G  G  P  E  V  P  F  H  P  G   118
GCAGAGAGGACAAGCCAGAGCCACCCCAGGAAGGACGTCTCCCTGATGCCACCAAGGGTT   480
  R  E  D  K  P  E  P  P  Q  E  G  R  L  P  D  A  T  K  G  C   138
GTGACCATTTGAGAGATGTCTTCATCAAGCAAATGGGTCTTACTGACCAGGACATTGTTG   540
  D  H  L  R  D  V  F  I  K  Q  M  G  L  T  D  Q  D  I  V  A   158
CTCTATCTGGAGGCCACACTTTGGGGAGATGCCACAAGGACCGCTCTGGTTTTGAAGGTG   600
  L  S  G  G  H  T  L  G  R  C  H  K  D  R  S  G  F  E  G  A   178
CTTGGACTACCAACCCTTTGGTCTTCGACAACACCTACTTCAAGGAGCTCCTGAGTGGTG   660
  W  T  T  N  P  L  V  F  D  N  T  Y  F  K  E  L  L  S  G  E   198
AGAAAGAAGGTCTCTTGCAGCTGCCATCTGACAAGGCTCTTCTCTCTGACCCTGTCTTCC   720
  K  E  G  L  L  Q  L  P  S  D  K  A  L  L  S  D  P  V  F  R   218
GCCCACTGGTTGAGAAATATGCAGCTGATGAAGATGCATTCTTTGCCGACTATGCTGAGG   780
  P  L  V  E  K  Y  A  A  D  E  D  A  F  F  A  D  Y  A  E  A   238
CGCACTTGAAACTTTCTGAACTCGGATTTGCTGATGCTTAAGCAGTTGGAGAAAGACTGA   840
  H  L  K  L  S  E  L  G  F  A  D  A  *                       250
TAGAAGGGTTGAAGAGAATAGCACCAGTGTCATCTTTTGTATTTCTTGCATTTACATGGG   900
GTTCGTTTAGTAACATTGCGCTTAGGTTGCTTTAGGGGAAGCTTGGTCATTTTAGTACCA   960
GGGCATCTAACTGTCTCGTTTGCTGAGTTTGATGGATTGTGGAAATGCATACTAGGATTT  1020
CTTTTTTGTGGATTGGTCATGATATTGATCATGTTGCTTGCTTTGGTAATAAAAGATGTT  1080
GAC                                                            1083
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Fig. 7.2. Nucleotide sequence and deduced amino acid sequence of SAP1(A) and SAP4 (B). The amino acid sequence deduced from an open reading frame is shown below the nucleotide sequences. The putative polyadenylation signal is shown by an underline. Putative N-glycosilation site, Asn-X-Ser/Thr, are double-underlined. Amino acid regions near the distal and proximal His are shaded. The nucleotide sequence data of SAP1 will appear in the DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D49679.

Fig. 7.3 compares the deduced amino acid sequences of SAP1, SAP4, *Arabidopsis* cytosolic AsAP (Kubo et al. 1992), and pea cytosolic AsAP (Mittler and Zilinskas 1991b). As a whole the two sequences of SAP1 and SAP4 were 50.8% identical. The deduced amino acid sequence of SAP1 was highly homologous with those of cytosolic AsAPs, 49.4% homologous with *Arabidopsis* AsAP and 47.8% homologous with pea AsAP. Considerable homology of cytosolic and chloroplastic AsAPs from higher plants has been found with yeast CCP in comparison with GP (Mittler and Zilinskas 1991b, Kubo et al. 1992, Chen et al. 1992). SAP4 was 35.1% identical with CCP in agreement

with the results of cytosolic AsAPs from pea and *Arabidopsis* (Mittler and Zilinskas 1991b, Kubo et al. 1992). SAP1 was also 30% identical over 250 amino acids with CCP and had less homology with the classical plant peroxidase.

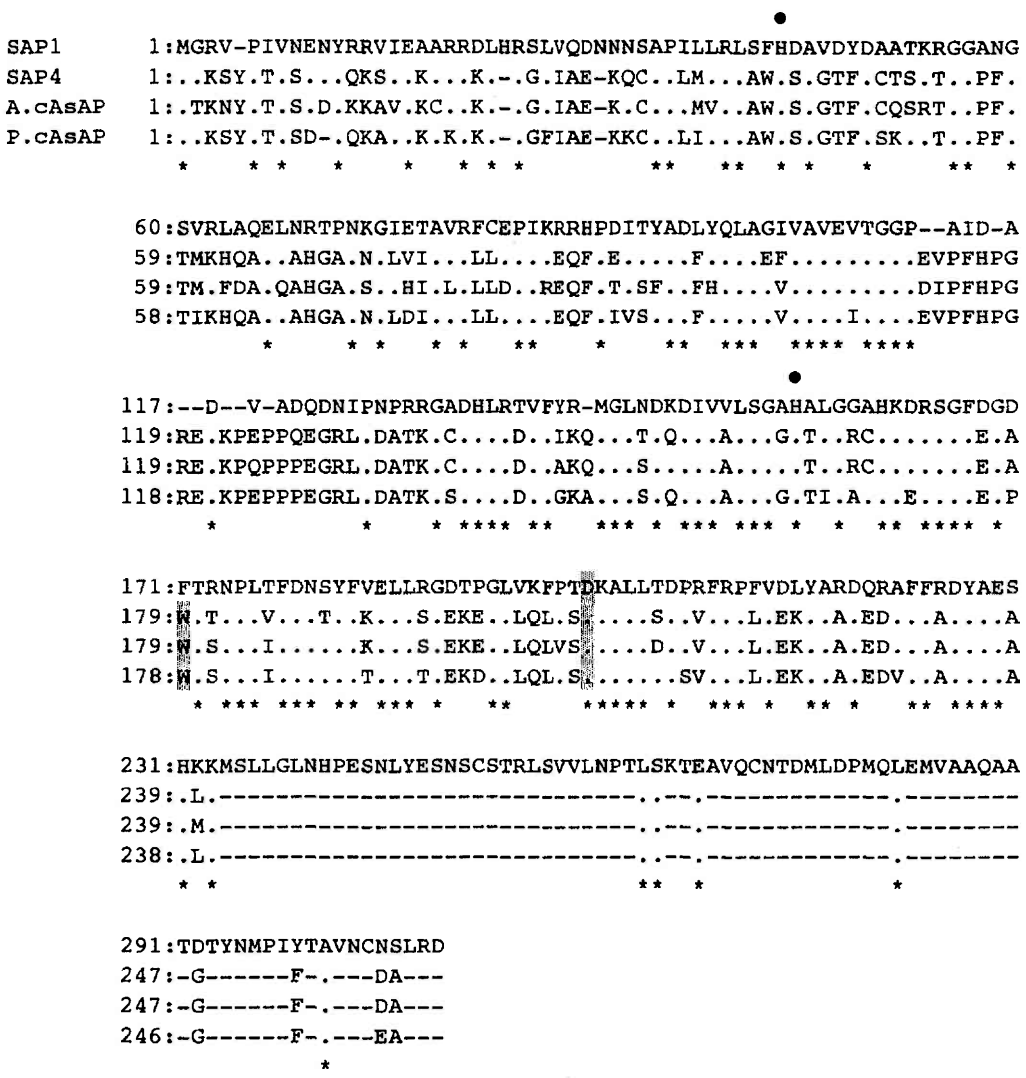

Fig. 7.3. Alignment of the deduced amino acid sequences of ascorbate peroxidases from spinach (SAP1 and SAP4) with the deduced amino acid sequences of cytosolic ascorbate peroxidases from *Arabidopsis* and pea. A.cAsAP, *Arabidopsis* cytosolic ascorbate peroxidase (Kubo et al. 1992); P.cAsAP, Pea cytosolic ascorbate peroxidase (Mittler and Zilinskas 1991b). Dashes are included to maximize alignment and dots indicate homology with the SAP1 amino acid sequence. The asterisks show the consensus amino acids. The distal and proximal His residues are shown by heavy dots. The Trp and Asp residue, which correlated with active site, are indicated by shade.

It is interesting to note that the C-terminal of SAP1 was approximately 60 amino acids longer than cytosolic AsAPs containing SAP4 (Fig. 7.3). As shown in Fig. 7.4, the 60-residue C-terminal region of SAP1 contained about 40% hydrophobic amino acids; the hydrophobicity in this region is higher than that in the region from N-terminus to residue 250, suggesting that SAP1 protein may be bound to the cell organelles as a peripheral membrane protein. There are two or three cytosolic AsAP isozymes, not including

chloroplastic isozymes, in spinach leaves (Tanaka et al. 1991, Nakano and Asada 1987). Moreover, Mittler and Zilinskas (1993) have reported that pea leaves contain several AsAP isozymes, which had different substrate specificities and enzymatic stabilities.

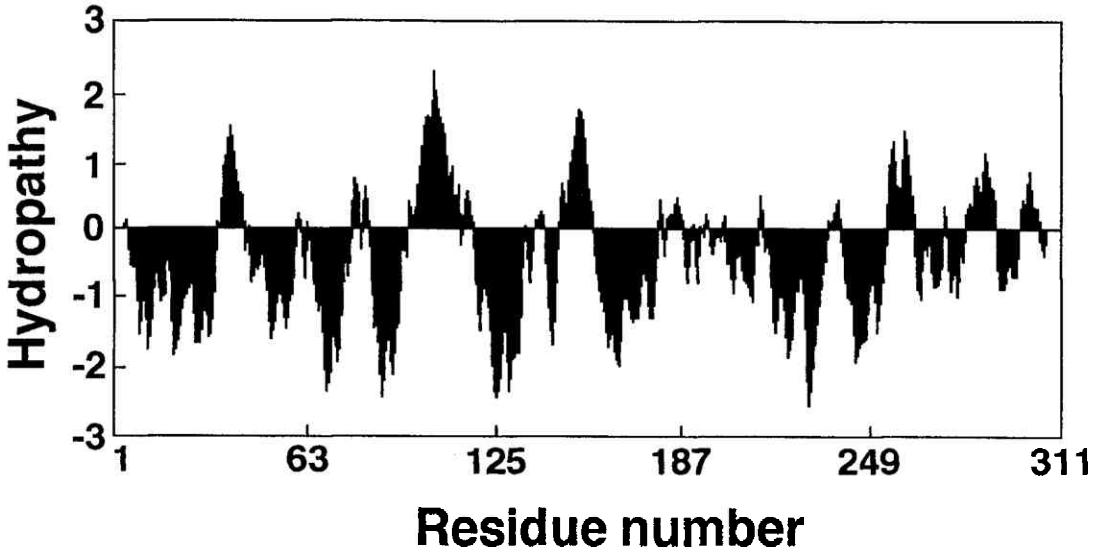

Fig. 7.4. Hydropathy profile of the deduced amino acid sequence of SAP1. Hydrophobicity was analyzed by the GENETYX software program, as described in reference (Kyte and Doolittle 1982), for a window size of nine amino acid residues. Hydrophobic domains are above the zero line.

		Distal His	Proximal His	
		•	•	
SAP1	36	ILLRLSFHDAVD	DKDIVVLSGAHALG	158
SAP4	35	LMLRLAWHSAGT	DQDIVALSGGHTLG	167
P.cAsAP	35	LILRLAWHSAGT	DQDIVALSGGHTIG	166
A.cAsAP	35	IMVRLAWHSAGT	DKDIVALSGAHTLG	166
Y.CCP	45	VLVRLAWHTSGT	DREVVALMGAHALG	177
HRP	35	SILRLHFHDCFV	SSDLVALSGGHTFG	173
TUP	35	SILRLFFHDCFV	TRDMVALSGAHTIG	171
LP(H8)	40	ESIRLVFHDSIA	EKELVWMLSAHSVA	179
MnP	39	EVIRLTFHDAIA	PFEVVSLLASHTVA	176

Fig. 7.5. Comparison of SAP1 and SAP4 from spinach with other peroxidases at regions near the distal and proximal His. P.cAsAP, pea cytosolic ascorbate peroxidase (Mittler and Zilinskas 1991b); A.cAsAP, *Arabidopsis* cytosolic ascorbate peroxidase (Kubo et al. 1992); Y.CCP, yeast cytochrome *c* peroxidase (Kaput et al. 1982); HRP, horseradish peroxidase (Welinder 1976); TUP, Turnip peroxidase (Mazza and Welinder 1980); LP (H8), lignin peroxidase H8 (Tien and Tu 1987); MnP, manganese-dependent peroxidase (Pease et al. 1989). Amino acids which are identical to SAP1 are shaded. Dots indicate distal and proximal His residues.

A comparison of the primary amino acid residues near the active site is shown in Fig. 7.5. Plant-type peroxidases contain two His residues, which are proposed to be essential for activity, one of the His residues (proximal) is the axial ligand of heme and the other one (distal) is in the distal pocket (Poulos and Kraut, 1980). The class I peroxidases are highly conserved around the distal His residues (R-L-A-W-H). SAP4

also had corresponding residues in the sequence. In contrast, SAP1 possessed Phe-42 rather than a Trp-42; thus in this respect, SAP1 was more similar to classical plant peroxidases than to cytosolic AsAPs. The amino acid sequence near the proximal His of cytosolic AsAPs was highly conserved in SAP1 and SAP4. The Asp-235 residue correlated with the active site of CCP (Fishel et al. 1991) was conserved in SAP1 and cytosolic AsAPs including SAP4 (Fig. 7.3). However, the active site Trp-191 in CCP which was important for the storage of oxidizing equivalents in compound I (Fishel et al. 1991), was not conserved in SAP1. The class II and III peroxidases are characterized by the eight Cys residues at the conserved positions for the four disulfide bonds (Welinder 1976). However, no half-cystine residues have been found in corresponding positions in either SAP1 or SAP4, as is the case for cytosolic AsAPs and CCP (Mittler and Zilinskas 1991b, Kubo et al. 1992, Kaput et al. 1982).

Most classical plant peroxidases are heavily glycosylated. Horseradish peroxidase possesses eight consensus *N*-glycosylation sites, Asn-X-Ser/Thr (Welinder 1976, Kornfeld and Korenfeld 1985). SAP1 contained four sites in the sequence at Asn residues 31, 58, 68, and 261 (Fig. 7.2.A). Neither AsAP nor CCP is glycolated (Asada 1992). As described below, SAP1 was expressed as a mature enzyme with a high AsAP activity in transformed *E. coli* cells. Accordingly, glycosylation of SAP1 protein may be low or lacking.

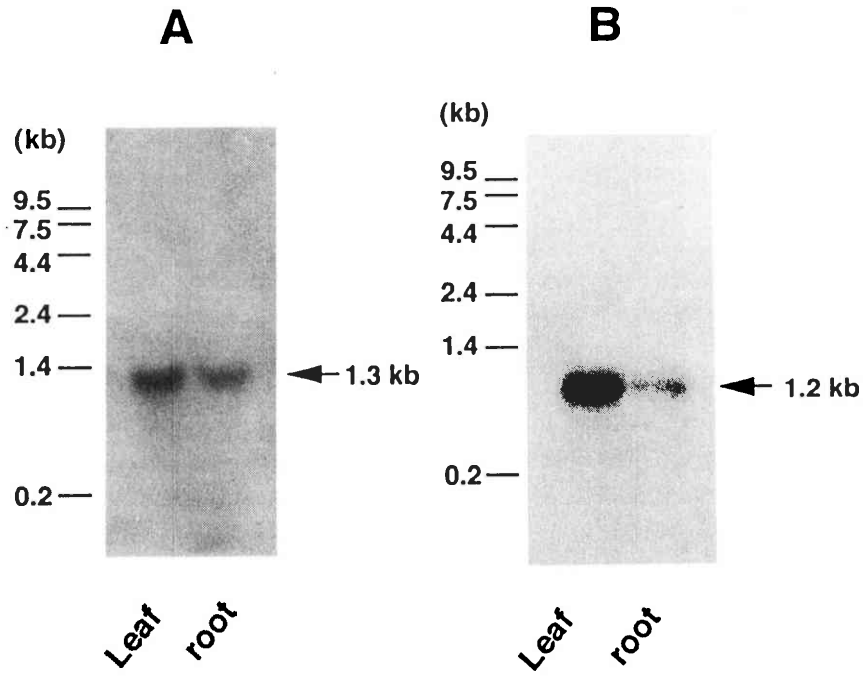

Fig. 7.6. Northern blot analysis of spinach leaf and root mRNA probed with the cDNA clones to the SAP1 (A) and SAP4 (B). Total RNA and mRNA were prepared from leaves and roots. In each lane of panel A, 20 μg of total RNA were put on. In panel B, 2 μg of poly(A)$^+$-RNA were put on. The migration of size markers is indicated on the left.

Northern blot analysis —— The expression of SAP1 mRNA as well as that of SAP4 was detected by Northern blot analysis using randomly primed cDNA inserts as hybridization probes. Each probe was hybridized to a 1.3-kb RNA of SAP1 and 1.2-kb of SAP4, respectively, indicating that the transcripts were found in both leaves and roots (Fig. 7.6). It has been reported that the pea cytosolic AsAP transcript, which was found to be 1050 b, was observed in both leaves and roots, and that steady state AsAP transcript levels increased in response to several stresses imposed by drought, heat, and paraquat (Mittler and Zilinskas 1992).

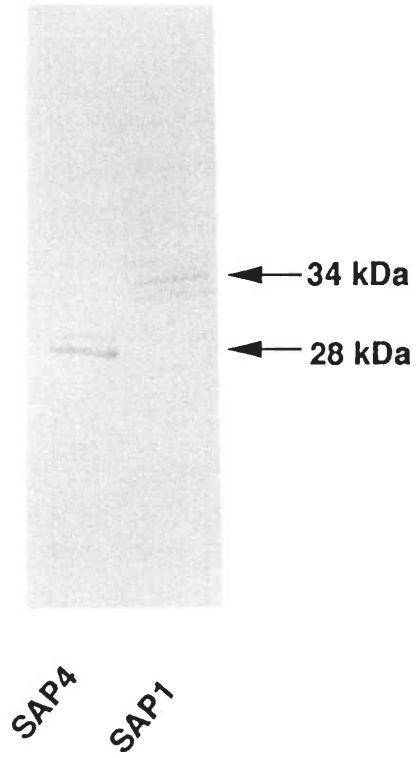

Fig. 7.7. Immunoblot analysis of crude extracts from *E. coli* cells that have been transformed with SAP1 and SAP4. Monoclonal antibody raised against *Euglena* AsAP was used as the primary antibody according to Chapter II. An arrowhead indicates the position of the protein recognized by the antibody.

Bacterial expression and AsAP activity —— After induction with IPTG, the recombinant AsAP proteins from SAP1 and SAP4 were expressed in *E. coli* cells. Western blot analysis was done with the monoclonal antibodies raised against *Euglena* AsAP, which reacted with the cytosolic AsAPs from plant sources, but did not crossreact with GP (Chapter II). As shown in Fig. 7.7, the antibodies reacted with a 34 kDa recombinant SAP1 protein and a 29 kDa recombinant SAP4 protein. Recombinant SAP1 correlated well with the calculated molecular mass of the primary protein. Some reports exist on the production of antibodies against plant AsAPs (Tanaka et al. 1991, Mittler and

Zilinskas 1991a, Koshiba 1993, Chen and Asada 1989). There is no evidence of cross-reaction with GP. These results suggest that SAP1 was homologous with AsAP, not GP.

AsAP is characterized by a specific electron donor for AsA, though the chloroplastic AsAP is more specific to AsA than the cytosolic enzyme (Asada 1992). Recently, William and Thomas (1994) have reported a recombinant pea cytosolic AsAP expressed and purified from *E. coli*, which has enzymatic and spectral properties nearly identical to the native AsAP. Recombinant SAP1 and SAP4 showed a high donor specificity for AsA and no or low activity for electron donors such as GSH, NADH, and guaiacol (Table 7.1), in agreement with native cytosolic AsAP from tea (Chen and Asada 1989) and pea (Mittler and Zilinskas 1991a) leaves. In contrast, GP purified from spinach leaves (Asada and Takahashi 1971), oxidized pyrogallol and guaiacol at higher rates than AsA (Table 7.1). Recombinant SAP1 did not reduce 1 mM t-butylhydroperoxide as an electron acceptor in the presence of AsA. These results indicate that the recombinant enzymes from SAP1 and SAP4 are clearly distinct from GP isozymes.

Table 7.1. Effects of different electron donors on the relative activity of the recombinant SAP1 (rSAP1), SAP4 (rSAP4), and endogenous guaiacol peroxidase (GP) from spinach. The preparation and assay of recombinant enzymes were done as described in "Materials and Methods."

Donor	rSAP1	rSAP4	GP
	Relative Activity (%)*		
AsA	100 (1.2)**	100 (3.6)	100 (1.0)
Iso-AsA	90	30	109
GSH	0	0	0
Cyt. *c*	0	0	0
NADPH	0	0	0
NADH	0	0	234
Pyrogallol	2370	308	46980
Guaiacol	64	10	9082

GSH : reduced glutathione, Cyt *c* : reduced cytochrome *c*
* The peroxidase activity for AsA was shown as 100% of activity.
**Specific activity (μmol min^{-1} mg $protein^{-1}$) given in parentheses

On the basis of the data reported here, I concluded that SAP1 can be classified as a new type of AsAP gene. Detailed studies on the detection and subcellular localization of endogeneous protein from the new SAP1 remain to be done.

Summary

Two cDNAs clones (SAP1 and SAP4) encoding ascorbate peroxidase were isolated from a cDNA library using the monoclonal antibodies raised against ascorbate peroxidase purified from *Euglena gracilis* Z. SAP1 contained an open reading frame encoding a protein of 309 amino acids and a calculated molecular mass of 34471 Da. The deduced amino acid sequence of SAP1 showd rather higher homology with the cytosolic ascorbate peroxidase from plant sources and SAP4 than with bacterial peroxidases and classical plant peroxidases. SAP4 contained an open reading frame encoding a protein of 250 amino acids and a calculated molecular mass of 27625 Da, in agreement with spinach cytosolic ascorbate peroxidase (Webb and Allen 1995). The complete coding sequence of the SAP1 and SAP4 were expressed in *E.coli*. The peroxidsae activity of recombinant SAP1 protein was 1.6-fold higher with ascorbate than with guaiacol, which was similar to those of SAP4. Northern blot analysis showed that ascorbate peroxidase mRNAs from SAP1 and SAP4 were expressed in leaves and roots. Here I conclude that SAP1 is a new type of cytosolic ascorbate peroxidase from spinach.

CHAPTER VIII

Cloning and Sequence Analysis of a cDNA Encoding Chloroplastic Ascorbate Peroxidase from Spinach

AsAP is known to have two types of isozymes, that is, chloroplastic and cytosolic forms (Asada 1992). In angiosperm chloroplasts, AsAP occurs in the stroma as a soluble form (sAsAP) and also in the thylakoids as a membrane-bound form (tAsAP). The enzymatic and molecular properties of AsAP isozymes have been characterized and are clearly different from those of guaiacol peroxidase (GP) from horseradish (Asada 1992).

Recently, manipulation of the expression of anti-oxidative enzymes including superoxide dismutase and glutathione reductase by gene transfer technology has provided new insights into the role of these enzymes in chloroplasts by allowing the direct investigation of their functions and interactions (Foyer et al. 1994b, Allen 1995). cDNAs for cytosolic AsAP (cAsAP), encoded by nuclear genes are isolated and characterized from many plant sources, including pea (Mittler and Zilinskas 1991b, 1992) and *Arabidopsis* (Kubo et al. 1992, 1993). However, no cDNAs that encode chloroplastic AsAPs have yet been identified. For this reason, it has not become clear how the regulation system of chloroplastic AsAP genes respond to environmental stress.

In Chapter VII, I reported two cDNA clones encoding a new type of cAsAP (SAP1) and have already determined cAsAP from mature spinach leaves using monoclonal antibodies raised against *Euglena* AsAP as a probe. Here, I describe the first complete cloning of a chloroplastic AsAP cDNA from spinach greening cotyledons using the *Euglena* mAb.

Materials and Methods

Materials —— Spinach seeds (*Spinacia oleracea*) were germinated on moist gauze at 15°C in the dark. The cotyledons from seedlings grown for 4-5 days in the dark were transferred to illumination (140 μE m^{-2} sec^{-1}) for 24 hr to obtain the greening cotyledons. All chemicals were reagent grade and used without further purification.

Construction and screening of cDNA library —— Total RNA was isolated from greening cotyledons of spinach seedlings (5.0 g, wet/wt) as described in Chapter VII. A cDNA was synthesized using a cDNA synthesis kit (Amersham, UK) and a cDNA library was constructed in λgt11 as described by the supplier (Amersham, UK). The spinach cDNA library in λgt11 was screened by monoclonal antibodies raised against *Euglena* AsAP (Chapter II). The cDNA insert from an immunopositive clone was

subcloned into the plasmid vector pBluescript SK(+) and used as a hybridization probe to obtain longer cDNA clones from the same library by plaque hybridization. The nucleotide of the longest cDNA clone was subcloned into pBluescript SK(+) and sequenced by the method as described in chapter VII. A cDNA clone containing the 5' end of the chloroplastic AsAP mRNA was obtained by 5' RACE PCR (Edwards et al. 1991) using nested antisense primers synthesized to sequences downstream of the N-terminal primer position.

Purification of stromal ascorbate peroxidase —— Intact chloroplasts were isolated from the fresh spinach leaves by percoll density centrifugation (Ishiko et al. 1992). The purification of sAsAP from intact chloroplasts was then done according to Nakano and Asada (1987). To determine the N-terminal amino acid sequence, partially purified sAsAP was separated by SDS-PAGE and the resolved proteins were electroblotted onto a PVDF membrane (Millipore, USA). The blot was stained in 0.1% Coomassie brilliant blue R-250 and the main band corresponding to the sAsAP protein (34 kDa) was cut out with a razor blade. The N-terminal sequences of the excised sAsAP band was performed by automated Edoman degradation using a model 477A sequencer (Applied Biosystems, USA).

Results and Discussion

In order to isolate a cDNA clone for putative chloroplastic AsAP, the *Euglena* anti AsAP mAbs (EAP1, EAP2), which cross-reacted with chloroplastic AsAP (Chapter II), was used as a probe.

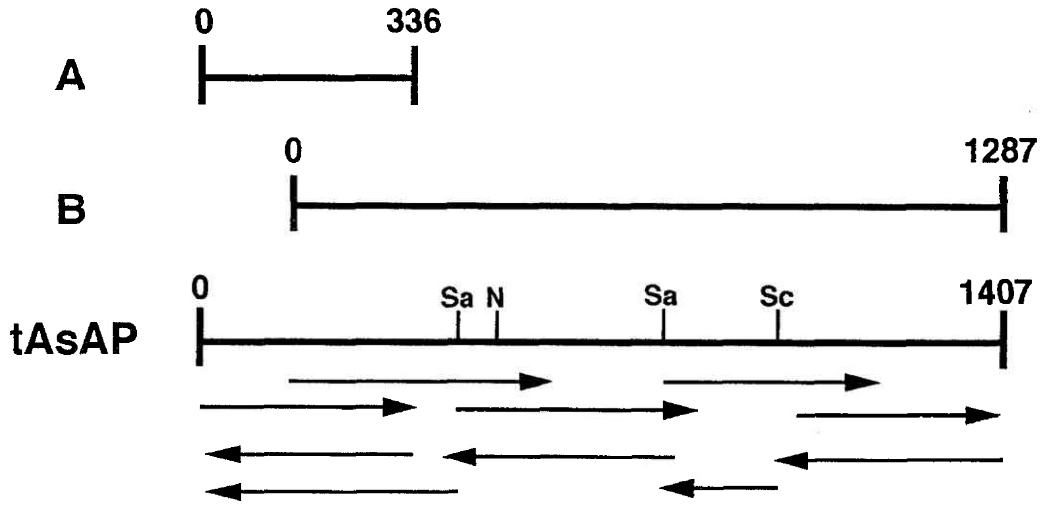

Fig. 8.1. Partial restriction map and cloning strategy of cDNA encoding thylakoid-bound AsAP (tAsAP) from spinach. tAsAP cDNA was constructed from the sequence of two overlapping cDNA clones A and B obtained by RACE-PCR using nested antisense primers synthesised to sequences at positions 317-336 bp and 372-391 bp downstream of the 5'-terminal position (see Fig. 8.2). Restriction enzymes are shown as follows: N, *Nde*I; Sa, *Sac*I; Sc, *Sca*I. Arrows indicate the direction and extent of sequencing.

```
GAAAAACCACCCAATCTCACTCACTTTCTCTCTCTATATTTTCAAACCACCACCCCGCAA   60
CCAATGACTGATCGTCTAGCAATGGCATCCTTCACTACCACCACCGCCGCCGCTGCATCT  120
                      M  A  S  F  T  T  T  T  A  A  A  A  S     13
CGTCTGCTTCCTTCTTCTTCCTCCTCCATCTCTCGACTTTCTCTCTCCTCTTCTTCCTCC  180
R  L  L  P  S  S  S  S  S  I  S  R  L  S  L  S  S  S  S  S     33
TCCTCCTCCTCACTCAAATGTCTCCGATCATCTCCACTCGTCTCTCACCTCTTCCTTCGA  240
S  S  S  S  L  K  C  L  R  S  S  P  L  V  S  H  L  F  L  R     53
CAGAGAGGAGGTTCAGCTTATGTGACGAAGACGAGGTTTAGCACGAAATGCTACGCTTCT  300
Q  R  G  G  S  A  Y  V  T  K  T  R  F  S  T  K  C  Y  A  S     73
                                                 ▲

GATCCTGCGCAGCTGAAGAATGCTAGGGAAGATATTAAAGAGCTTCTTCAATCTAAGTTC  360
D  P  A  Q  L  K  N  A  R  E  D  I  K  E  L  L  Q  S  K  F     93
TGTCATCCTATTATGGTTCGCTTAGGTTGGCACGATGCCGGTACTTATAATAAGGACATT  420
C  H  P  I  M  V  R  L  G  W  H  D  A  G  T  Y  N  K  D  I    113
AAAGAATGGCCACAAAGAGGTGGAGCCAATGGCAGTCTGAGCTTTGATGTTGAGCTCAGG  480
K  E  W  P  Q  R  G  G  A  N  G  S  L  S  F  D  V  E  L  R    133
CATGGAGCTAATGCAGGTCTTGTTAATGCCCTGAAACTTCTACAGCCCATAAAAGACAAG  540
H  G  A  N  A  G  L  V  N  A  L  K  L  L  Q  P  I  K  D  K    153
TACTCTGGAGTTACATATGCAGATCTATTCCAGCTGGCTAGTGCTACTGCAATAGAGGAG  600
Y  S  G  V  T  Y  A  D  L  F  Q  L  A  S  A  T  A  I  E  E    173
GCTGGTGGTCCAACAATACCCATGAAGTATGGAAGAGTGGATGCCACAGGGCCGGAGCAG  660
A  G  G  P  T  I  P  M  K  Y  G  R  V  D  A  T  G  P  E  Q    193
TGCCCAGAAGAAGGAAGGCTTCCTGATGCTGGACCTCCTTCACCTGCTCAACATCTACGT  720
C  P  E  E  G  R  L  P  D  A  G  P  P  S  P  A  Q  H  L  R    213
GATGTTTTCTACAGAATGGGTCTTGATGATAAGGATATAGTAGCATTATCTGGAGCACAT  780
D  V  F  Y  R  M  G  L  D  D  K  D  I  V  A  L  S  G  A  H    233
ACGTTGGGAAGGTCTAGACCTGAACGCAGTGGTTGGGGCAAGCCAGAGACTAAATACACG  840
T  L  G  R  S  R  P  E  R  S  G  W  G  K  P  E  T  K  Y  T    253
AAAGATGGACCTGGAGCTCCAGGAGGGCAGTCATGGACTGCGGAGTGGTTGAAGTTTGAT  900
K  D  G  P  G  A  P  G  G  Q  S  W  T  A  E  W  L  K  F  D    273
AATTCCTATTTCAAGGACATCAAAGAAAAGAGAGATGCAGATTTGCTTGTTTTGCCAACT  960
N  S  Y  F  K  D  I  K  E  K  R  D  A  D  L  L  V  L  P  T    293
GATGCTGCTCTTTTCGAAGATCCGTCTTTCAAGGTATATGCAGAGAAATATGCAGCTGAC 1020
D  A  A  L  F  E  D  P  S  F  K  V  Y  A  E  K  Y  A  A  D    313
CAAGAAGCATTTTTCAAGGATTACGCTGAAGCCCATGCCAAACTCAGCAACCAAGGAGCC 1080
Q  E  A  F  F  K  D  Y  A  E  A  H  A  K  L  S  N  Q  G  A    333
AAATTTGACCCTGCTGAGGGTATCACTCTTAATGGAACCCCTGCCGGAGCAGCTCCAGAG 1140
K  F  D  P  A  E  G  I  T  L  N  G  T  P  A  G  A  A  P  E    353
AAGTTTGTAGCAGCCAAGTACTCATCTAACAAGAGATCAGAGCTTTCGGATTCTATGAAG 1200
K  F  V  A  A  K  Y  S  S  N  K  R  S  E  L  S  D  S  M  K    373
GAAAAGATTCGCGCTGAATATGAAGGTTTTGGAGGTAGCCCTAATAAGCCTCTACCAACA 1260
E  K  I  R  A  E  Y  E  G  F  G  G  S  P  N  K  P  L  P  T    393
AACTACTTCCTAAACATTATGATTGTGATTGGAGTTTTGGCAGTTCTATCATATCTTGCG 1320
N  Y  F  L  N  I  M  I  V  I  G  V  L  A  V  L  S  Y  L  A    413
GGAAATTGATTTGTGGTTTGATGAGTTTTTTTCCATTTATAAATATAACGGCAGTTGATT 1380
G  N  *                                                       415
ATATGAAAAAAAAAAAAAAAAAAAA                                     1407
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Fig. 8.2. Nucleotide sequence of spinach thylakoid-bound AsAP and deduced amino acid sequences. The amino acid sequence deduced from an open reading frame is shown below the nucleotide sequences. The arrow indicates the putative cleavage site of the transit peptide. The putative *N*-glycosilation site, Asn-X-Ser/Thr, are underlined. The nucleotide sequence data reported in this chapter will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession number D77997.

A 950 bp cDNA fragment was isolated from a λgt11 cDNA library constructed with mRNA isolated from the greening cotyledons of spinach seedlings. The same cDNA

library was rescreened by plaque hybridization using the 950 bp cDNA fragment as a probe. One clone with a 1287 bp-length insert was selected for sequence analysis, which lacked the 5' end containing the putative start codon. We employed 5'RACE to amplify the unknown sequence at the 5' end of the cDNA clone. The resulting 336 bp fragment was found to contain 216 bp of overlapping sequence with the former clone. The whole nucleotide sequence consisted of 1407 bp (Fig. 8.1). It is not possible to unambiguously deduce the translational start for the spinach chloroplastic AsAP-precursor protein. The open reading frame preceding the mature polypeptide includes two in-phase methionine residues at nucleotide positions 64-66 and 82-84. The sequences contiguous with these, the first methionine codons, match the consensus sequences for plant translation initiation sites (A/GA/CX ATG G) with purine at -3 and G at position +4 being the most critical (Lutcke et al. 1987). So, I assumed that the ATG codon at position 82-84 acts as the initiator codon. The complete sequence revealed the open reading frame of 1248 bp encoding 415 amino acids (Fig. 8.2). The calculated molecular mass of the encoded protein was 45015 Da.

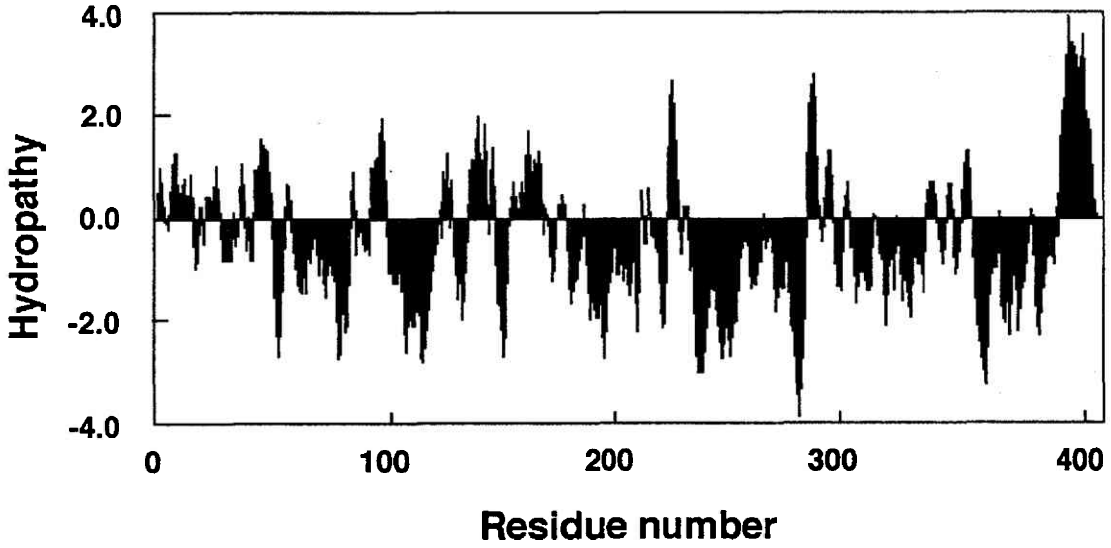

Fig. 8.3. Hydropathy profile of the deduced amino acid sequence of spinach thylakoid-bound AsAP. Hydrophobicity was analyzed by the GENETYX software program, as described Kyte and Doolittle (1982), for a window size of nine amino acid residues. The hydrophobic domains are above the zero line.

The corresponding sequence with the purified tAsAP from spinach leaves was found in the first 20 amino acid sequence of the deduced amino acids 71 to 90 (Miyake et al. 1993). So, the position of deduced amino acids between 70 and 71 was defined as the cleavage site. The calculated molecular mass of the predicted mature protein was 37710 Da. The tAsAP solubilized and purified from spinach leaves was estimated to be a molecular mass of 40000 ± 2000 Da by gel-filtration (Miyake et al. 1993). Furthermore, hydropathy analysis showed that the predicted mature protein has one major hydrophobic

region (residues 380-415) at the C-terminous domain, which may be the domain for the binding to the thylakoid membranes (Fig. 8.3). These results clearly indicate that the isolated cDNA clone encodes tAsAP of the spinach chloroplasts.

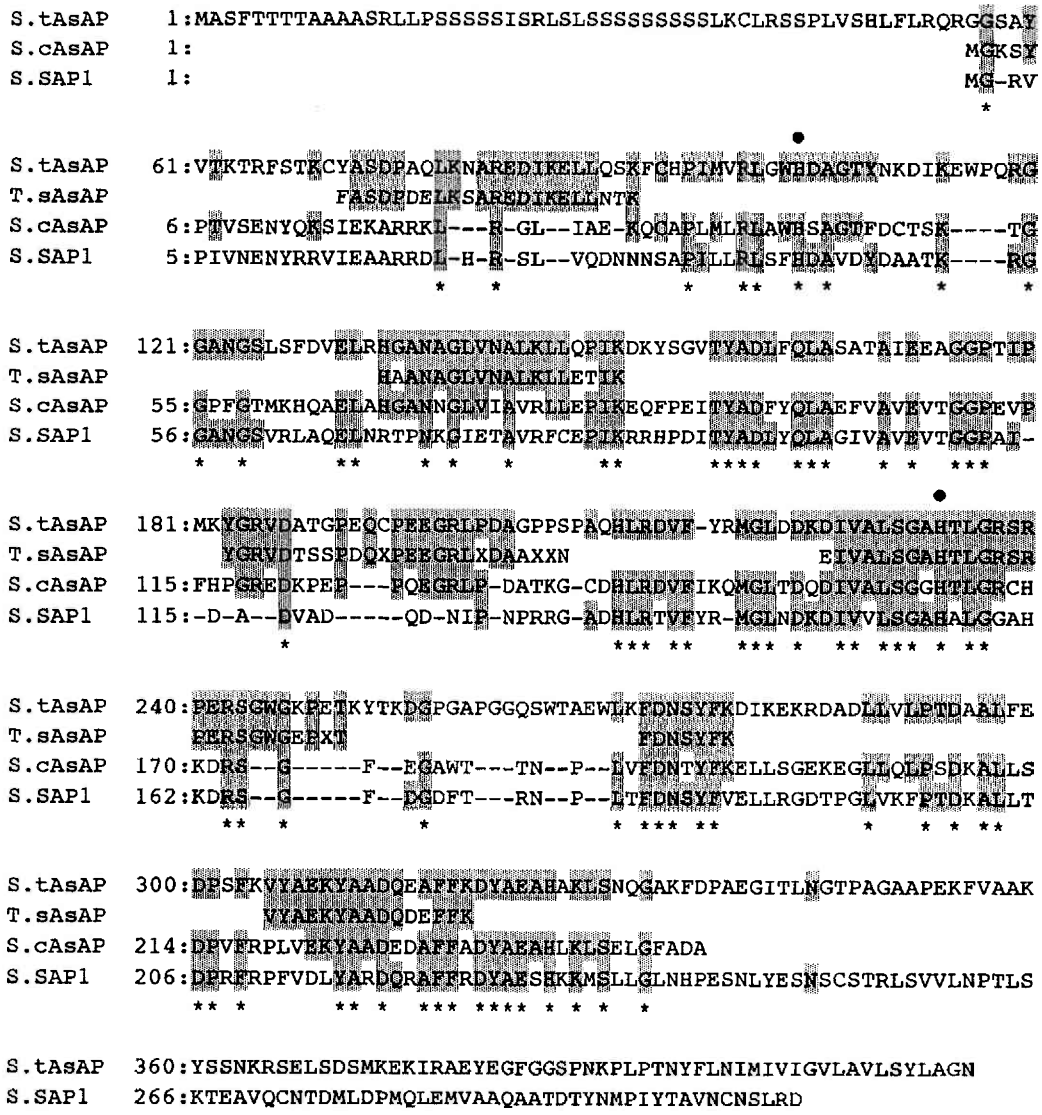

Fig. 8.4. *Alignment of the deduced amino acid sequences of spinach thylakoid-bound AsAP with* the partial amino acid sequence of tea stromal AsAP and the deduced amino acid sequences of spinach cytosolic AsAPs. S.tAsAP, spinach thylakoid-bound ascorbate peroxidase (this study); T.sAsAP, tea stromal ascorbate peroxidase (Chen et al. 1992); S.cAsAP, spinach cytosolic ascorbate peroxidase (Chapter VII); S.SAP1, spinach cytosolic ascorbate peroxidase isozyme (Chapter VII). Amino acids, which are identical to the spinach thylakoid-bound ascorbate peroxidase, are shaded. Dashes are included to maximize alignment. The asterisks show the consensus amino acids. The distal and proximal His residues are shown by heavy dots.

The sequence upstream of the N-terminus of the mature AsAP protein encoded 70 amino acid residues, with a predicted molecular mass of 7322 Da, which had several features common to most chloroplastic transit peptides. Like the transit peptides of the majority of chloroplastic precursors, the transit peptide of the tAsAP precursor contained few acidic residues, was rich in serine and threonine, and had a net positive charge. It

also had a potential to form an amphiphilic β-strand close to the putative processing site (de Boer and Weisbeek 1991).

Fig. 8.4 compares the predicted amino acid sequence encoding spinach tAsAP with those of other AsAPs. The tAsAP from spinach does exhibit a highly significant homology (82.4%) over a 108 residual region to partial amino acid sequences of sAsAP purified from tea leaves (Chen et al. 1992). The deduced spinach tAsAP had a 46.5% and 40.2% homology with that of cAsAP and SAP1 from spinach, respectively. Moreover, the deduced spinach tAsAP protein showed 46.5% amino acid identity with the *Arabidopsis* cAsAP (Kubo et al. 1992) and 43.8% identity with pea cAsAP (Mittler and Zilinskas 1992). AsAP and yeast cytochrome *c* peroxidase (CCP) have been classified as a member of the class I plant peroxidase from its amino acid sequence and has been part of the lineage of prokaryotic peroxidases (Welinder 1992). The deduced spinach tAsAP was 34.6% identical over 240 amino acids with yeast CCP and had less homology with the classical plant peroxidase such as GP. This is also the case for the other previously described AsAPs (Kubo et al. 1992, Chen et al. 1992, Chapter VII).

In order to analyze the relationships among plant type peroxidases, a phylogenic tree was constructed according to Hein's alignment algorithm (Hein 1990). As illustrated in Fig. 8.5, tAsAP was more closely related to the cAsAPs and yeast CCP than to other peroxidases. This result clearly supports the fact that tAsAP belongs to be the class I peroxidase as well as cAsAPs. Such a high degree of homology among AsAPs suggests that AsAP genes might have evolved from the same ancestral gene and have been far from classical plant peroxidase such as GP in terms of molecular evolution.

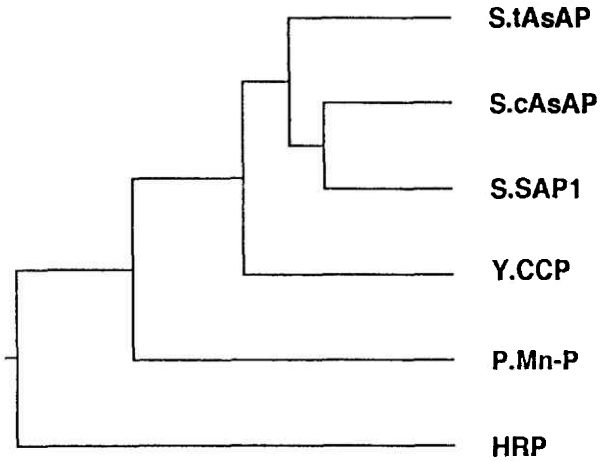

Fig. 8.5. Phylogenic tree for spinach thylakoid-bound and cytosolic AsAPs and other plant type peroxidases. The dendrogram was generated by comparison of the known amino acid sequences according to Hein (1990). The length of the branches is proportional to the evolutionary divergence. S.tAsAP, spinach thylakoid-bound ascorbate peroxidase (this study); S.cAsAP, spinach cytosolic ascorbate peroxidase (Chapter VII); S.SAP1, spinach cytosolic ascorbate peroxidase isozyme (Chapter VII); Y.CCP, yeast cytochrome *c* peroxidase (Kaput et al. 1982); P.Mn-P *Phanerochaete chrysosporium* manganese-dependent peroxidase (Pease et al. 1989); HRP, horseradish peroxidase (Welinder 1976).

Distal (His-104) and proximal (His-233) histidine residues at the active site are indicated in Fig. 8.4 by dots. The class I peroxidases share the common features of the distal histidine site (R-L-A-W-H). The tAsAP also had highly conserved residues in the sequence, except for one residue at the position of glycine-102. The proximal histidine site of the tAsAP agreed very closely with that of tea sAsAP (Fig. 8.4). The tAsAP contained two putative *N*-glycosilation sites which follow the general rule of Asn-X-Thr/Ser (Kornfeld and Korenfeld 1985). Tea sAsAP shows that its sugar content is lower than 0.25% and is not a glycoprotein (Chen and Asada 1989). The putative *N*-glycosylation sites of spinach tAsAP may not be glycolated.

In order to make comparisons with the primary structures of the chloroplastic AsAP isozymes, sAsAP was purified from isolated intact spinach chloroplasts. The sAsAP showed a molecular mass of 34000 Da as a monomer judged by gel-filtration and SDS-PAGE. The amino acid sequence of the N-terminal residues of the mature sAsAP from spinach was determined as follows: YASDPAQLKNAREDIKELLQ. Interestingly, the first 20 amino acids of spinach sAsAP exhibited a complete consensus sequence with that of spinach tAsAP. Chen and Asada (1989) reported the molecular mass of tea sAsAP was estimated to be 34000 Da and had approximately 309 amino acids deduced from its amino acid composition. From the amino acids position 71 to 379 of the predicted tAsAP, which corresponded to that of tea sAsAP, it is possible to calculate the molecular mass of 33885 Da. Furthermore, the amino acid composition of spinach tAsAP determined from its predicted amino acids (residues 71-379) was similar to that of tea sAsAP (data not shown). The residual 36 amino acids residues of the C-terminal region (residues 380-415) had a hydrohobic domain as described above. Recently, we isolated the cDNA encoding a new type of cAsAP (SAP1), which is different from that of the already known cAsAP (Chapter VII). Six AsAP isozymes were found in bell peppers and a comparison of these isozymes showed differences in its growth conditions (Schantz et al. 1995). A novel AsAP isozyme was found to be localized on the membranes of microbodies in pumpkin (Yamaguchi et al. 1995). Taking into account the data reported so far and the present findings, it seems likely that the AsAP of spinach is a multigene family and there are at least more than three AsAP genes, two of which may be encoded by nearly identical chloroplastic genes, differing only in the presence or absence of the 3' coding regions, which construct a hydrophobic region at the C-terminus domain in the AsAP protein.

Summary

A cDNA clone encoding thylakoid-bound ascorbate peroxidase was isolated from a spinach cDNA library constructed by greening cotyledons from seedlings using the monoclonal antibody raised against *Euglena* ascorbate peroxidase as a probe. The cDNA contained an open reading frame encoding a mature protein of 345 amino acids with a calculated molecular mass of 37,710 Da preceded by a transit peptide of 70 amino acid residues. The deduced amino acid sequence had 40-46% and 34.6% homology to the other known ascorbate peroxidases from plant sources and cytochrome *c* peroxidase from yeast, respectively.

CHAPTER IX

Conclusion

In *Euglena gracilis*, which lacks catalase, the antioxidant enzymes involved in the AsA-GSH cycle occur only in the cytosol but not in the chloroplasts. The use of this organism as a model species for studying the effect of altered levels of AsAP involved in protection against oxidative stress has meaned that a better knowledge is required of AsAP and of its regulation.

Euglena AsAP was purified to homogeneity. Although the *Euglena* AsAP showed closely similar enzymlogical properties to those of cytosolic AsAP isozyme from higher plants, the enzyme possessed its own properties; the high molecular weight and the diference of amino acid sequence of the N-terminus. Especially, it is worth noting that the *Euglena* AsAP also reduced *t*-butyl hydroperoxide and cumene hydroperoxide as an electron acceptor in the presence of AsA. To my knowledge, *Euglena* AsAP is the first AsAP whose substrate is an organic hydroperoxide as well as H_2O_2 in photosynthetic eukaryotes. In Chapter III, the AsAP activity was not observed in Fe-deficient *Euglena* cells, and that lipid peroxides (thiobarbituric acid-reactive substances) in Fe-deficient cells were approximately 2.6-fold greater than those in Fe-sufficient cells. These results suggest that the *Euglena* AsAP, like glutathione peroxidase in animals and *Chlamydomonas*, may serve to protect the cell membrane by reducing the peroxide compounds generated endogenously from unsaturated fatty acids. N-terminal amino acid sequence of *Euglena* AsAP showed no significant similarity to any other AsAPs from higher plants. From the result of partial amino acid sequences of *Euglena* AsAP, however, the enzyme exhibited a high degree of homology to sequences of cytosolic and chloroplastic AsAPs in higher plants, suggesting that *Euglena* AsAP gene is also comprised in the same ancestral gene.

Chapter III and Chapter IV show that both Fe and light become important regulatry factors for AsAP expression. Fe-deficient *Euglena* cells are good model to investigate the molecular mechanism of AsAP expression. The AsAP activity was not found in Fe-deficient cells as stated above. The addition of Fe to the Fe-deficient cells causes the AsAP activity to increase by two phase, the former was the activation of AsAP and the later was de novo synthesis of AsAP protein. Illumination of dark-grown *Euglena* cells caused an increase in the AsAP activity. The activities of enzymes related to AsA-GSH cycle such as SOD, MDAsA reductase, DAsA reductase, and GSH reductase were also found to rise parallel to the increase in AsAP activity. The increased level of the enzyme activities in AsA-GSH cycle were attributed to synthesis de novo of their proteins. These

facts clearly indicate that elevated levels of antioxidative components can be considered an early physiological response of *Euglena* cells to remove H_2O_2 generated *in vivo* .

It is an interesting problem why the *Euglena* cells localize AsAP only in the cytosol, but not in the chloroplasts, to scavenge H_2O_2 generated *in vivo*. Chapter V demonstrated that H_2O_2 formed in *Euglena* chloroplasts and mitochondria diffuses from these organelles into the cytosol. On the basis of these observations, Fig. 9.1 showed the working models of protective system against H_2O_2 in *Euglena* and higher plant cells. In higher plants, H_2O_2 is eliminated by AsAP located in chloroplasts and cytosol and catalase located in microbodies. On the other hand, in *Euglena* cells, H_2O_2 generated in chloroplasts and mitochondria must diffuse from each organelle into the cytosol and then decompose by the AsA-GSH cycle including AsAP. The diffusion of H_2O_2 from chloroplasts into the cytosol as well as the H_2O_2-scavenging by AsAP seems to be a protective system against oxidative stress in *Euglena* cells.

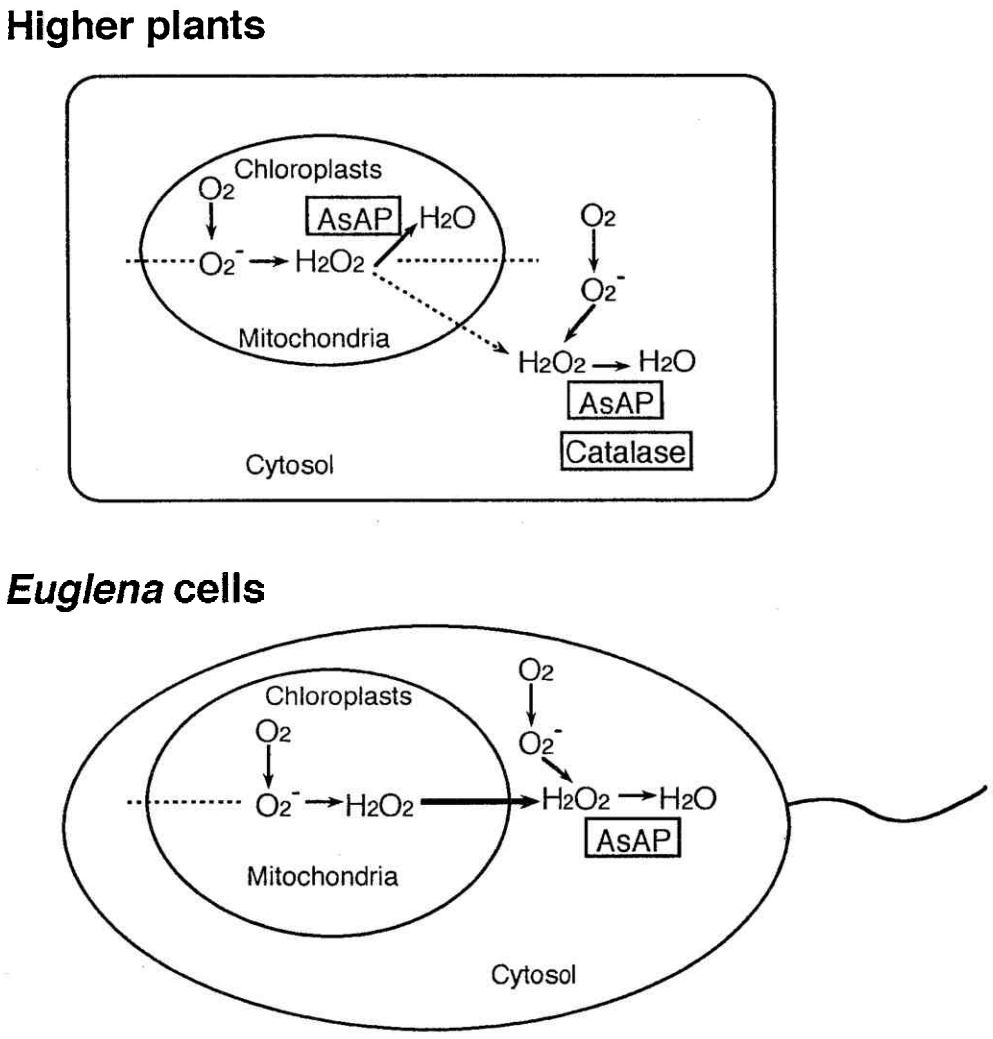

Fig. 9.1 Metabolism of H_2O_2 in higher plants and *Euglena* cells.

In higher plants, the situation to induce the response of antioxidative enzymes to environmental stresses is often considerably complicated by the presence of a large number of isoenzyme forms. For example, AsAP is located in chloroplasts, microbodies and cytosol. In komatsuna (*Brassica Rapa*) leaves, chloroplastic AsAP was a low content (approximately 20%) compared with cytosolic AsAP and komatsuna leaves contain only one type of cytosolic isozyme. It is likely that komatsuna is also the useful plant model to investigate the response of cytosolic AsAP to alterations in the oxidative stresses.

At the present time, only one type of cytosolic AsAP is cloned and no cDNAs that encode chloroplastic AsAPs have yet been identified. For this reason, little is known about the molecular mechanism of AsAP isozymes underlying their response to environmental stresses. One of monoclonal antibodies (EAP1) raised against purified *Euglena* AsAP cross-reacted with both cytosolic and chloroplastic AsAP isozymes in higher plants, indicating that *Euglena* AsAP is immunologically related to AsAP isozymes in higher plant and that EAP1 become a good probe to screen a cDNA encoding AsAP isozymes from higher plants. Actually, Chapter VII and Chapter VIII showed cDNA cloning of cDNAs encoding two cytosolic AsAP isozymes (SAP1 and SAP4) and a cDNA encoding thylakoid-bound AsAP of spinach leaves using EAP1 as a probe. SAP1 was identified to be a new type of AsAP isozyme. Comparison of the deduced amino acid sequences of three AsAP isozymes showed approximately 45% identity among these isozymes, suggested that AsAP isozyme in spinach is a multigene family. These cDNAs and monoclonal antibodies prepared in this study would provide excellent probes for studying the molecular mechanism of AsAP genes response to environmental stresses.

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