

cDNAs encoding spinach stromal and thylakoid-bound ascorbate peroxidase, differing in the presence or absence of their 3'-coding regions**

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Abstract Two cDNA clones encoding stromal (SAP28) and thylakoid-bound (SAP22) ascorbate peroxidase were isolated from a spinach cDNA library constructed by greening cotyledons. The SAP22 and SAP28 contained an open reading frame encoding mature protein of 295 and 345 amino acids with calculated molecular mass of 32 239 Da and 37 710 Da, respectively, preceded by the common transit peptides of 70 amino acid residues. Interestingly, the N-terminal 364 amino acids of SAP22 were 100% identical with SAP28 except for one C-terminal amino acid residue (Asp-365), and the C-terminal of SAP22, which is the putative transmembrane segment, was 50 amino acids longer than that of SAP28.

Key words: Ascorbate peroxidase; Isozyme; cDNA cloning; Chloroplast; Expression; Spinach (*Spinacia oleracea*)

1. Introduction

Ascorbate peroxidase (AsAP; EC 1.11.1.11) plays a major role in preventing the accumulation of toxic levels of hydrogen peroxide in higher plants and algae including *Euglena* [1,2]. AsAP is known to have two types of isozymes, i.e. chloroplastic and cytosolic forms [1]. In angiosperm chloroplasts, which lack catalase and classical plant peroxidases such as guaiacol peroxidase (GP), 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 GP from horseradish [1].

Recently, manipulation of the expression of antioxidative 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 [3,4]. cDNAs for cytosolic AsAP (cAsAP) encoded by nuclear genes have been isolated and characterized from many plant sources, including pea [5] and *Arabidopsis* [6]. However, the lack of information on chloroplastic AsAP cDNA has limited

the understanding of the regulation system of chloroplastic AsAP genes.

In a previous study, we reported that one of the monoclonal antibodies (mAb; EAP1) raised against *Euglena* AsAP cross-reacted with both cytosolic and chloroplastic AsAP isozymes in spinach leaves [7]. This indicates that EAP1 should be a good probe to screen a cDNA encoding AsAP isozyme from spinach. Actually, we obtained two cDNA clones encoding a new type of cytosolic AsAP (SAP1) and have already determined cAsAP from mature spinach leaves using EAP1 as a probe [8].

Here, we describe the first complete cloning of sAsAP and tAsAP cDNAs from spinach greening cotyledons using the *Euglena* AsAP mAb. We suggest that chloroplastic AsAP isozymes are encoded by nearly identical gene, differing only in the presence or absence of a C-terminal peptide, which constructs a hydrophobic thylakoid membrane binding domain.

2. Materials and methods

2.1. 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 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 24 h to obtain the greening cotyledons. All chemicals were reagent grade and used without further purification.

2.2. Construction and screening of cDNA library

Total RNA was isolated from greening cotyledons of spinach seedlings (5.0 g wet weight) with guanidine thiocyanate, and poly (A)⁺RNA was purified using the PolyAtract mRNA Isolation System (Promega, USA). A cDNA was synthesized using a cDNA synthesis kit (Amersham, UK). The spinach cDNA library constructed in λ gt11 was screened by mAb raised against *Euglena* AsAP [7]. The cDNA insert from an immunopositive clone was subcloned into the plasmid vector pBluescript SK(+) and used as a hybridization probe to obtain the longest cDNA clones from the same library by plaque hybridization. The nucleotide of the isolated cDNA clones was sequenced by a dideoxynucleotide dye primer method with a Model 373A DNA Sequencer (Applied Biosystems, USA).

2.3. Production of recombinant chloroplastic ascorbate peroxidases

The tAsAP (SAP22) and sAsAP (SAP28) cDNAs were expressed in the pET expression system of Studier et al. [9]. To subclone the mature tAsAP protein coding region in the pET3a expression vector, the cDNA was amplified by PCR using two mutagenic primers (N-terminus; 5'-TTTAGCACGCATATGTACGCTTCTGATCC-3' and C-terminus; 5'-ACTCATGGATCCACAAATCAATTCCCCGC-3'; the underlined bases are the mutations relative to the original cDNA sequence) to introduce an *Nde*I site at the initiation codon. The cDNA encoding sAsAP underwent mutagenesis using the C-terminal primer (5'-TGGATCCTTTAATCCTTGTTAGATG-3'; the underlined bases are the mutations relative to the original cDNA sequence) and an N-terminal primer which was the same as that of the mature tAsAP protein. The *Nde*I-*Bam*HI tAsAP and sAsAP cDNA fragments were cloned into the *Nde*I-*Bam*HI sites of the pET3a vector desig-

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**The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers, D77997 (tAsAP) and D83669 (sAsAP).

Abbreviations: AsA, ascorbate; AsAP, ascorbate peroxidase; CCP, cytochrome *c* peroxidase; GP, guaiacol peroxidase; mAb, monoclonal antibody; cAsAP, cytosolic ascorbate peroxidase; sAsAP, stromal ascorbate peroxidase; tAsAP, thylakoid-bound ascorbate peroxidase.

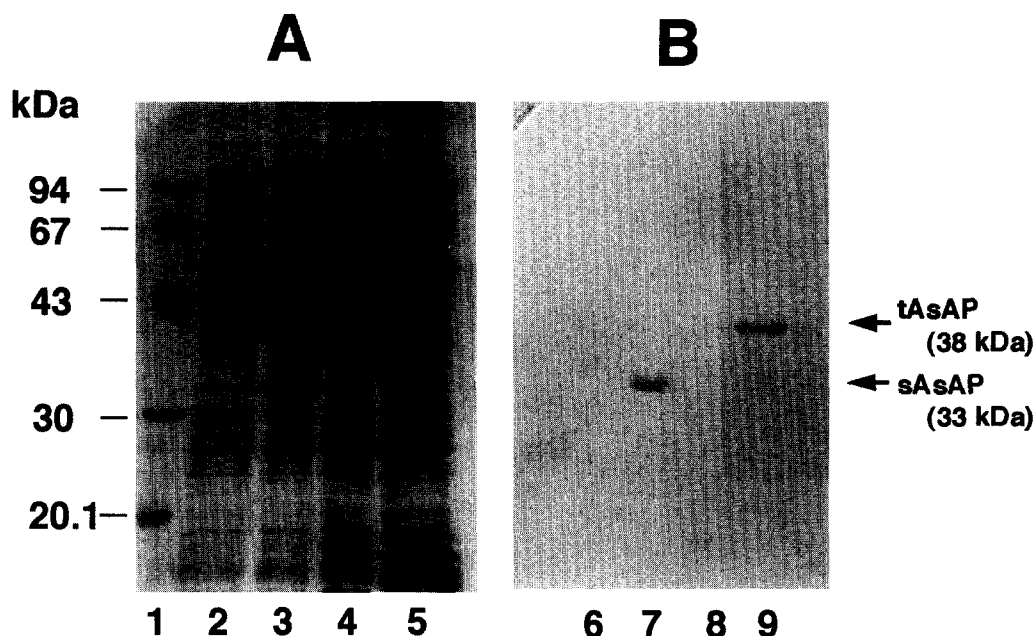


Fig. 2. Expression of the mature sAsAP and tAsAP in *E. coli*. Protein samples were separated by SDS-PAGE (12.5% gels) and stained with Coomassie blue (A) or analyzed by immunoblotting (B) using *Euglena* AsAP mAb. Lanes: 1, molecular mass markers; 2,6, total soluble extract of pET3a-transformed *E. coli*; 3,7, total soluble extract of pETsAsAP-transformed *E. coli*; 4,8, solubilized insoluble fraction of pET3a-transformed *E. coli*; 5,9, solubilized insoluble fraction of pETtAsAP-transformed *E. coli*.

gesting that the processing site in the precursor protein is between residue 70 and 71. Therefore, the mature SAP28 protein contained 295 amino acid residues with a molecular mass of 32 239 Da, which is in good agreement with the value estimated for the purified spinach sAsAP of 33 ± 1 kDa. The predicted amino acid sequences of the sAsAP clone agreed completely with the sequences of the endogenous 8 peptides from the native sAsAP shown by the underlining in Fig. 1.

The recombinant mature SAP28 protein, which correlated with the deduced molecular mass from SAP28 ORF, was expressed at higher levels than bacterial proteins (Fig. 2, lane 3). The *E. coli* enzyme showed an AsAP activity of $17.8 \mu\text{mol/min per mg protein}$ and was highly specific to AsA as the electron donor, like the spinach sAsAP [11] (data not shown). The immunoblot of the lysate from mature SAP28-expressing cells using the *Euglena* mAb EAP1 [7] was characterized by a predominant band at 33 kDa (Fig. 2, lane 7). No cross-reactivity was observed with an *E. coli* extract from pET3a-transformed cells lacking the SAP28 insert (Fig. 2, lane 6).

The sequence upstream of the N-terminus of the mature SAP28 protein encoded 70 amino acid residues, with a predicted molecular mass of 7322 Da, which had several features common to most chloroplastic transit peptides [13]. The transit peptide of the SAP28 precursor contained few acidic residues, was rich in Ser and Thr, and had a net positive charge. It also had the potential to form an amphiphilic β -strand close to the putative processing site [13]. These results demonstrated that the SAP28 encodes sAsAP.

The insert of SAP22 contained 1407 bp (Fig. 1). The complete sequence revealed the ORF of 1248 bp encoding 415 amino acids with a molecular mass of 45 015 Da (Fig. 1). Interestingly, the N-terminal 364 amino acids of SAP22 were 100% identical with sAsAP except for one C-terminal amino acid residue (Asp-365), and the C-terminal of SAP22 was 50 amino acids longer than sAsAP. The calculated mole-

cular mass of the predicted mature protein of SAP22 was 37,710 Da, which is in good agreement with the molecular mass of 40 ± 2 kDa for the tAsAP solubilized and purified from spinach leaves [14]. The corresponding sequence of the N-terminus to the 21st residue of the native tAsAP [14] was found in the deduced amino acids 71–91. Furthermore, hydrophathy analysis showed that the predicted mature protein of SAP22 has one major hydrophobic region (residues 380–415) at the C-terminus domain, which may be the domain for the

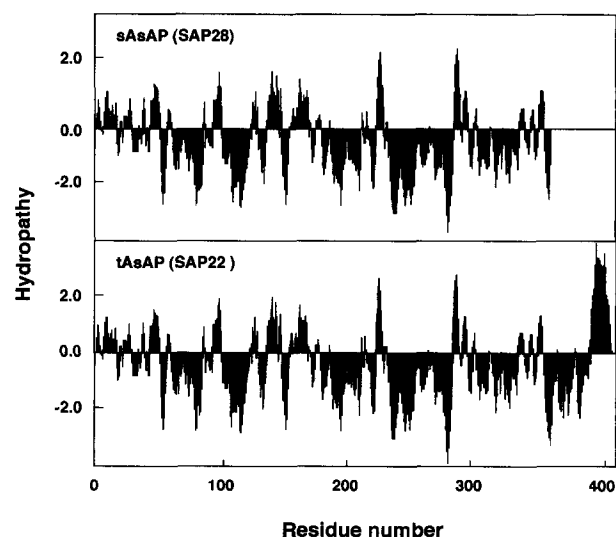


Fig. 3. Hydropathy profile of the deduced amino acid sequence of spinach sAsAP and tAsAP. Hydropathy was analyzed by the GENETYX software program, as described in [25], for a window size of nine amino acid residues. The hydrophobic domains are above the zero line.

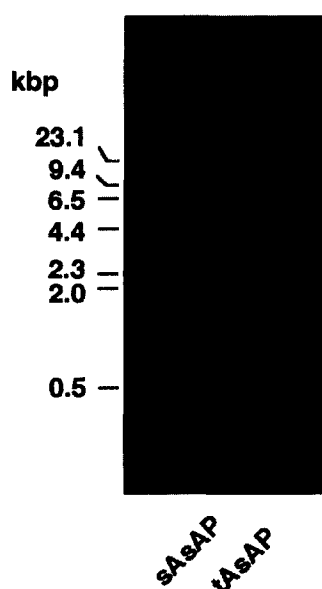


Fig. 4. RT-PCR analysis of transcripts from spinach greening cotyledons. PCR proceeded as described in section 2, and the products were analyzed by electrophoresis on a 1% agarose gel followed by staining with ethidium bromide.

binding to the thylakoid membranes (Fig. 3). When the recombinant mature tAsAP was expressed in *E. coli*, the accumulation of the recombinant protein could be detected in the inclusion bodies (Fig. 2, lane 5). The *Euglena* mAb EAP1 cross-reacted with the recombinant mature SAP22 solubilized from the inclusion bodies, which correlated with the predicted molecular mass of 38 kDa. We therefore conclude that the SAP22 encodes tAsAP of the spinach chloroplasts.

The predicted amino acid sequences encoding spinach sAsAP and tAsAP were compared with those of other AsAPs. The chloroplastic AsAPs from spinach exhibited a highly significant homology (82.4%) over 108 amino acids with the partial amino acid sequences of sAsAP purified from tea leaves [15]. Both the deduced spinach sAsAP and tAsAP showed homologous sequences with spinach cAsAP (46.5%) [8], spinach SAP1 (40.2%) [8], *Arabidopsis* cAsAP (46.5%) [6] and pea cAsAP (43.8%) [5]. AsAP and yeast cytochrome *c* peroxidase (CCP) have been classified as members of the class I plant peroxidases from their amino acid sequences and have been part of the lineage of prokaryotic peroxidases [16]. Both deduced spinach sAsAP and tAsAP were 34.6% identical over 240 amino acids with yeast CCP and had less homology with the classical plant peroxidases such as GP. This is also the case for the other previously described AsAPs [5,6,8].

The class I peroxidases share the common features of the distal His site (R-L-A-W-H). The chloroplastic AsAPs (His-104) also had highly conserved residues in the sequence, except for one residue at the position of Gly-102. The proximal His site (His-233) of the chloroplastic AsAPs agreed very closely with that of tea sAsAP [15]. The Asp-294 residue correlated with the active site of CCP [17] was conserved in chloroplastic AsAPs. The Trp residue, which is correlated to the storage of oxidizing equivalents in compound I [17], exists at the amino acid position of 265. No half-Cys residues were found in the chloroplastic AsAPs, as is the case for cAsAPs and CCP [6,8,18].

With RT-PCR using the primers at the common 5'-region and at the respective specific 3'-region of SAP28 and SAP22, we attempted to analyze the expression of chloroplastic AsAP isozyme mRNAs in spinach greening cotyledons. The PCR reaction products revealed the single expected size band pattern (sAsAP; 1332 bp, tAsAP; 1301 bp) on agarose gel electrophoresis (Fig. 4), indicating that sAsAP- and tAsAP-related transcripts are expressed in spinach greening cotyledons.

The data presented here indicate that chloroplastic AsAP isozymes differ only in the presence or absence of the 3'-coding regions, which construct a hydrophobic region at the C-terminus domain in the tAsAP protein. Recently, we isolated the cDNA encoding a new type of cAsAP (SAP1), which is different from that of the already known cAsAP [7]. Six AsAP isozymes have been found in bell peppers and a comparison of these isozymes showed differences in growth conditions [19]. A novel AsAP isozyme was found to be localized on the membranes of microbodies in pumpkin and cotton [20,21]. Accordingly, it seems likely that the AsAP of spinach is a multigene family and there are at least more than four AsAP genes, two of which may be encoded by nearly identical chloroplastic genes. However, the finding that the cDNAs encoding chloroplastic AsAP isozymes either contained or lacked a sequence with an intact reading frame leads us to suggest the possibility that spinach chloroplastic sAsAP and tAsAP are encoded by only one gene, which may generate mRNAs for chloroplastic AsAP isozymes from the primary transcripts by alternative usage of the final distinct two exons. The production of heterogeneity in the amino acid sequences by alternative splicing is well documented in animal systems leading to proteins which are development- and tissue-specific [22]. Recently, in *Drosophila*, it has been reported that three mRNA species encoding aldolase isozymes are generated through one of the three 3'-terminal exons including one genomic gene [23]. In higher plants, the two isozymes of spinach ribulosebiphosphate carboxylase/oxygenase activase have arisen from alternative splicing of a common pre-mRNA, in which the mRNAs near the 3'-end of the coding region differed by the presence or absence of the 22-bp sequence [24]. Clarification of genomic DNA encoding chloroplastic AsAP isozymes from spinach is now underway in our laboratory.

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References

- [1] Asada, K. (1992) *Physiol. Plant.* 85, 235–241.
- [2] Shigeoka, S., Nakano, Y. and Kitaoka, S. (1980) *Biochem. J.* 186, 377–380.
- [3] Foyer, C.H., Descourvières, P. and Kunert, K.J. (1994) *Plant Cell Environ.* 17, 507–523.
- [4] Allen, R.D. (1995) *Plant Physiol.* 107, 1049–1054.
- [5] Mittler, R. and Zilinskas, B.A. (1991) *FEBS Lett.* 289, 257–259.
- [6] Kubo, A., Saji, H., Tanaka, K., Tanaka, K. and Kondo, N. (1992) *Plant Mol. Biol.* 18, 691–701.
- [7] Ishikawa, T., Kohno, H., Takeda, T. and Shigeoka, S. (1996) *Biochim. Biophys. Acta*, in press.
- [8] Ishikawa, T., Sakai, K., Takeda, T. and Shigeoka, S. (1995) *FEBS Lett.* 367, 28–32.

- [9] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) *Methods Enzymol.* 185, 60–89.
- [10] Ishiko, H., Shigeoka, S., Nakano Y. and Mitsunaga, T. (1992) *Phytochemistry* 31, 1499–1500.
- [11] Nakano, Y. and Asada, K. (1987) *Plant Cell Physiol.* 28, 131–140.
- [12] Lutcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F. and Sceelee, G.A. (1987) *EMBO J.* 6, 43–48.
- [13] De Boer, A.D. and Weisbeek, P.J. (1991) *Biochim. Biophys. Acta* 1071, 221–253.
- [14] Miyake, C., Cao, W.-H. and Asada, K. (1993) *Plant Cell Physiol.* 34, 881–889.
- [15] Chen, G.-X., Sano, S. and Asada, K. (1992) *Plant Cell Physiol.* 33, 109–116.
- [16] Welinder, K.G. (1992) *Curr. Opin. Struct. Biol.* 2, 388–393.
- [17] Fishel, L.A., Farnum, M.F., Maruo, J.M., Miller, M.A. and Kraut, J. (1991) *Biochemistry* 30, 1986–1996.
- [18] Kaput, J., Goltz, S. and Blobel, G. (1982) *J. Biol. Chem.* 257, 15054–15058.
- [19] Schantz, M.-L., Schreiber, H., Guillemaut, P. and Schantz, R. (1995) *FEBS Lett.* 358, 149–152.
- [20] Yamaguchi, K., Mori, H. and Nishimura, M. (1995) *Plant Cell Physiol.* 36, 1157–1162.
- [21] Bunkelmann, J.R. and Trelease, R.N. (1996) *Plant Physiol.* 110, 589–598.
- [22] Breitbart, R.E., Andreadis, A. and Nadal-Ginard, B. (1987) *Annu. Rev. Biochem.* 56, 467–495.
- [23] Kai, T., Sugimoto, Y., Kusakabe, T., Zhang, R., Koga, K. and Hori, K. (1992) *J. Biochem.* 112, 677–688.
- [24] Werneke, J.M., Chatfield, J.M. and Organ, W.L. (1989) *Plant Cell* 1, 815–825.
- [25] Kyte, J. and Doolittle, R.F. (1982) *J. Mol. Biol.* 157, 105–132.