Cloning and expression of cDNA encoding a new type of ascorbate peroxidase from spinach

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Abstract A cDNA clone (SAP1) encoding a peroxidase was isolated from a spinach cDNA library using monoclonal antibodies raised against *Euglena* ascorbate peroxidase. The deduced amino acid sequence of SAP1 had higher homology with the cytosolic ascorbate peroxidases from plant sources than with bacterial peroxidases and classical plant peroxidases. The peroxidase activity of recombinant SAP1 protein expressed in *E. coli* was 1.6-fold higher with ascorbate than with guaiacol, which was similar to those of endogenous cytosolic ascorbate peroxidases. Here we conclude that SAP1 belongs to a new type of ascorbate peroxidase from spinach.

Key words: Ascorbate peroxidase; Peroxidase; cDNA cloning; Expression; Spinach

1. Introduction

Plant-type peroxidases have been grouped in three classes, which are distantly related structurally [1]. Class I is formed by prokaryotic lineages such as yeast CCP and AsAP. Secretory fungal peroxidases (manganese and lignin peroxidases) belong to Class II, while the classical plant peroxidases such as GP belong to class III.

AsAP is widely distributed in higher plants and algae including *Euglena* [2,3] and serves to rid cells of excess hydrogen peroxide generated under normal and stress conditions [4,5]. AsAP has been known as three type of isozymes, that is, thylakoid-bound, stromal, and cytosolic forms [2]. The enzymatic and molecular properties of AsAP have been characterized and are clearly different from those of GP from horseradish [2]. The organization of the cDNA clones coding the cytosolic AsAP has been reported in pea [6], *Arabidopsis* [7], soybean [8], maize [9], and spinach (Webb and Allen, accession number L20864). Here we report the cloning and expression in *E. coli* of the full-length cDNA clone encoding a new type of AsAP from spinach.

2. Materials and methods

2.1. Materials

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

2.2. Screening of the cDNA library

A spinach cDNA library in λZAP was screened by monoclonal antibodies raised against *Euglena* AsAP. The purification of *Euglena* AsAP and monoclonal antibodies preparation will be described elsewhere. 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.

2.3. Preparation of recombinant protein

E. coli cells were grown with shaking at 37°C to an OD_{600} of 0.6–0.7 in LB medium containing 100 μg/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 $500 \times g$ 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 $10,000 \times g$ for 15 min was used as the crude recombinant enzyme.

2.4. Assay of peroxidase activity

AsAP was assayed by the decrease in A at 290 nm due to ascorbate (2.8 $mM^{-1} \cdot cm^{-1}$) oxidation [10]. The reaction mixture contained 50 mM potassium phosphate, pH 7.0, 0.4 mM AsA, 1 mM EDTA, and 0.1 mM H_2O_2. 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 $mM^{-1} \cdot cm^{-1}$) or 20 mM guaiacol (460 nm, 11.3 $mM^{-1} \cdot cm^{-1}$).

2.5. Northern blot analysis

Total RNA was extracted from leaves and roots of spinach as described in reference [11]. 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, pSAP1 and pSAP4 were used as each probes. The membranes were washed in 0.2 × standard saline citrate/0.1% SDS at 60°C for 20 min.

3. Results and discussion

3.1. Isolation and characterization of cDNA encoding ascorbate peroxidase

The spinach cDNA library was screened with the monoclonal antibodies raised against *Euglena* AsAP. Two positive clones, SAP1 and SAP4, containing inserts of 1.2-kb and 1.1-kb, respectively, were isolated and completely sequenced. 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 34,471 Da (Fig. 1). On the other hand, the 1.1-kb insert of SAP4 contained an ORF coding for a protein of 250 amino acid

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the Accession Number D49697.

Abbreviations: AsA, Ascorbate; AsAP, Ascorbate peroxidase; CCP, Cytochrome *c* peroxidase; GP, Guaiacol peroxidase IPTG, Isopropyl-β-D-thiogalactopyranoside.

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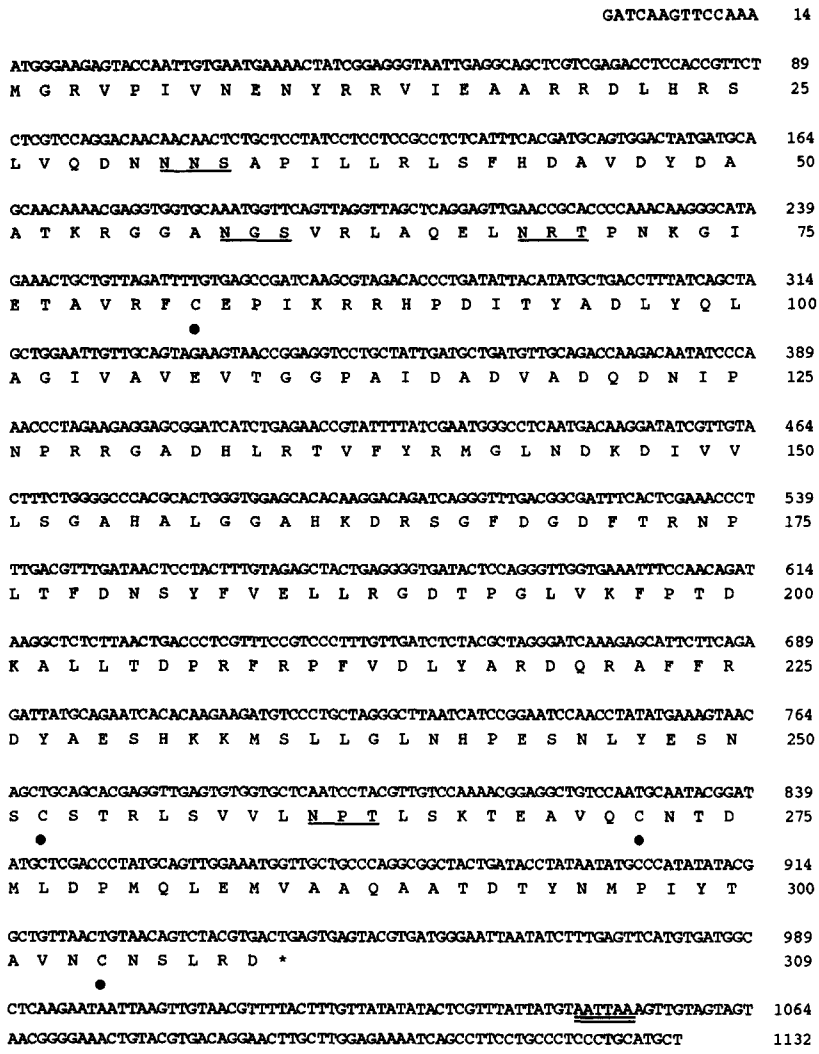

Fig. 1. Nucleotide sequence of SAP1 and deduced amino acid sequences. The amino acid sequence deduced from an open reading frame is shown below the nucleotide sequences. The putative polyadenylation signal is shown by a double underline. Cys residues are shown by heavy dots. Putative N-glycosylation site, Asn-X-Ser/Thr, are underlined.

residues, which was calculated to be 27,560 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, GenBank Accession Number L20864), 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. [12]. In contrast, the deduced N terminus of SAP1 was little homologous with cytosolic AsAPs including SAP4 and the classical plant peroxidases [13,14,15].

Fig. 2 compares the deduced amino acid sequences of SAP1, SAP4, *Arabidopsis* cytosolic AsAP [7], and pea cytosolic AsAP [6]. 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 [6,7,16]. SAP4 was 35.1% identical with CCP in agreement with the results of cytosolic AsAPs from pea and *Arabidopsis* [6,7]. SAP1 was also 30% identical over 250 amino acids with CCP and had less homology with the classical plant peroxidase. It is interesting to note that the C-terminal of SAP1 was approximately 60 amino acids longer than cytosolic AsAPs containing SAP4 (Fig. 2). 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 [12,17]. Moreover, Mittler and Zilinskas have reported that pea leaves contain several AsAP isozymes, which had different substrate specificities and enzymatic stabilities [18].

A comparison of the primary amino acid residues near the active site is shown in Fig. 3. 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 [19]. The class

Fig. 2. 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. AAP, *Arabidopsis* ascorbate peroxidase [7]; PAP, Pea ascorbate peroxidase [6]. 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 shading.

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 [20] was conserved in SAP1 and

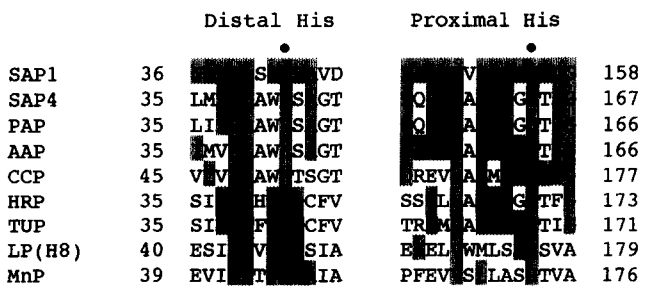

Fig. 3. Comparison of SAP1 and SAP4 from spinach with other peroxidases at regions near the distal and proximal His. PAP, pea ascorbate peroxidase [6]; AAP, *Arabidopsis* ascorbate peroxidase [7]; CCP, yeast cytochrome *c* peroxidase [21]; HRP, horseradish peroxidase [15]; TUP, Turnip peroxidase [28]; LP (H8), lignin peroxidase H8 [29]; MnP, manganese-dependent peroxidase [30]. Amino acids which are identical to SAP1 are shaded. Dots indicate distal and proximal His residues.

Table 1
Effects of different electron donors on the relative activity of the recombinant SAP1 (rSAP1), SAP4 (rSAP4), and endogenous guaiacol peroxidase (GP) from spinach

Donor	Relative activity (%)*		
	rSAP1	rSAP4	GP
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	2,370	308	46,980
Guaiacol	64	10	9,082

The preparation and assay of recombinant enzymes were done as described in section 2.
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.

cytosolic AsAPs including SAP4 (Fig. 2). However, the active site Trp^{191} in CCP which was important for the storage of oxidizing equivalents in compound I [20], 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 [15]. 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 [6,7,21].

Most classical plant peroxidases are heavily glycosylated. Horseradish peroxidase possesses eight consensus N-glycosylation sites, Asn-X-Ser/Thr [15,22]. SAP1 contained four sites in the sequence at Asn residues 31, 58, 68, and 261 (Fig. 1). Neither AsAP nor CCP is glycolated [2]. 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.

3.2. 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. 4). It has been reported that the pea cytosolic AsAP transcript, which was found to be 1,050 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 [23].

3.3. 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 (Ishikawa et al., unpublished data). As shown in Fig. 5, 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.

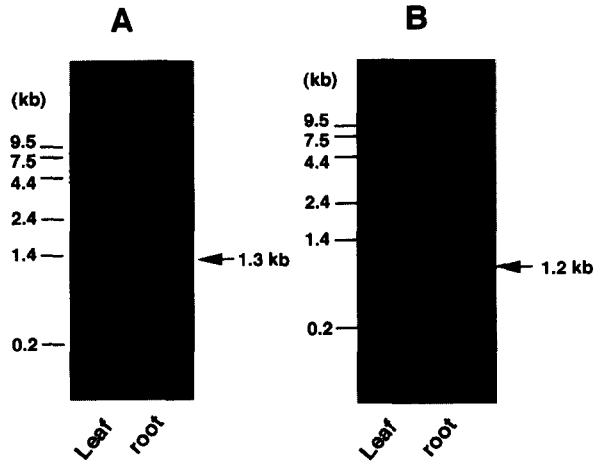

Fig. 4. Northern blot analysis of spinach leaf and root mRNA probed with the cDNA clones to the SAP1 (A) and SAP4 (B). Total RNA and mRNA were prepared from leaves and roots. In each lane of panel A, 20 μg of total RNA were put on. In panel B, 2 μg of poly(A)$^+$-RNA were put on. The migration of size markers is indicated on the left.

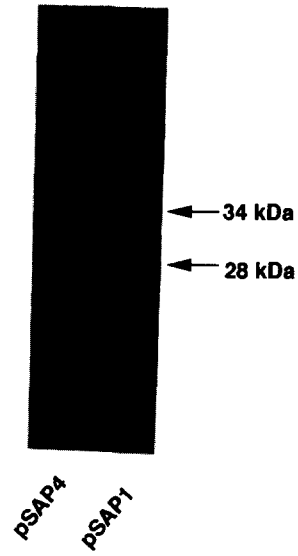

Fig. 5. Immunoblot analysis of crude extracts from *E. coli* cells that have been transformed with pSAP1 and pSAP4. Monoclonal antibody raised against purified *Euglena* AsAP was used as the primary antibody. An arrowhead indicates the position of the protein recognized by the antibody.

Some reports exist on the production of antibodies against plant AsAPs [12,13,14,24]. 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 [2]. Recently, William and Thomas 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 [25]. Recombinant SAP1 and SAP4 showed a high donor specificity for AsA and no or low activity for electron donors such as GSH and NADH (Table 1), in agreement with native cytosolic AsAP from tea [24] and pea [13] leaves. When AsA was the electron donor, 1 mM *t*-butylhydroperoxide was not used as an electron acceptor (data not shown). In contrast, GP purified from spinach leaves [26], oxidized pyrogallol and guaiacol at higher rates than AsA (Table 1). These results indicate that the recombinant enzymes from SAP1 and SAP4 are clearly distinct from GP isozymes.

On the basis of the data reported here, we conclude 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.

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