

Molecular cloning and nucleotide sequence analysis of a cDNA encoding pea cytosolic ascorbate peroxidase

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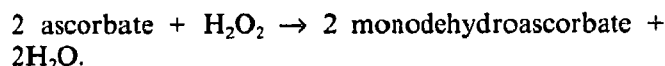
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A cDNA clone encoding the cytosolic ascorbate peroxidase of pea (*Pisum sativum* L.) was isolated and its nucleotide sequence determined. While ascorbate peroxidase shares limited overall homology with other peroxidases, significant homology with all known peroxidases was found in the vicinity of the putative active site.

Ascorbate peroxidase; Cytochrome *c* peroxidase; Horseradish peroxidase; cDNA clone; *Pisum sativum*

1. INTRODUCTION

Aerobic organisms possess enzymes capable of removing toxic intermediates of oxygenic metabolism. Catalase, peroxidases, and superoxide dismutases are such enzymes functioning to scavenge the toxic forms of oxygen [1]. Although the molecular properties of many of these enzymes are well-established, no structural information exists regarding the key enzyme that participates in the detoxification pathway of H_2O_2 in higher plants namely, ascorbate peroxidase (APX). The physiological significance of ascorbate peroxidase was first noted by Foyer and Halliwell [2], and the enzyme has been purified to homogeneity or near-homogeneity from several sources [3–6]. Ascorbate peroxidase exists as two distinct isozymes, chloroplastic and cytosolic, both of which catalyze the reaction:



Each isozyme possesses a distinct preference for ascorbate as an electron donor. The importance of APX in providing a means to rid cells of excess H_2O_2 is suggested by the increase in APX activity in response to several environmental stress conditions [7,8]. We have recently purified to homogeneity cytosolic APX from pea shoots [6]. The current report is the first describing the molecular cloning and complete amino acid sequence of an ascorbate peroxidase. We hope that this

report will contribute to further molecular studies of this key enzyme in detoxification of reactive oxygen in plant tissues.

2. MATERIALS AND METHODS

2.1. Screening of the λ gt11 library

A cDNA library, constructed from pea leaf mRNA [9] in the expression vector λ gt11, was generously provided by Dr J. Stephen Gantt (University of Minnesota).

The library was screened according to [10], using the *Escherichia coli* host strain Y1090. Plaques expressing proteins which cross-reacted with polyclonal, affinity-purified antibodies raised against pea cytosolic APX, were detected enzymatically with goat anti-rabbit IgG conjugated to alkaline phosphatase. Purification of pea cytosolic APX and antibody preparation will be described elsewhere [6].

2.2. Isolation of a cDNA Encoding Ascorbate peroxidase

Recombinant λ gt11 DNA was isolated according to [11] and subsequently digested with *Eco*RI to release the cDNA insert. The restriction fragments were separated by electrophoresis in 1% Seakem (FMC) agarose gel and eluted and purified using GeneClean (Bio101). The 1 kb fragments were cloned into pBluescript KS- (Stratagene), which had been linearized with *Eco*RI. Transformation of *E. coli* XL1B (Stratagene) was carried out according to [12].

2.3. DNA Sequence Analysis

Plasmid DNA was isolated by the alkaline lysis method [13]. The cDNA inserts cloned into pBluescript were sequenced by the dideoxy chain termination method [14], using modifications for double-stranded plasmid templates [15]. Sequencing reactions were carried out with T-7 Polymerase (Pharmacia) with $[\alpha\text{-}^{35}\text{S}]\text{dATP}$ as the radiolabeled nucleotide. Synthetic 17-base primers, complementary to the plasmid cloning vector and to internal sites in the cDNA insert, synthesized on a Milligen Biosearch Cyclone instrument, were used to prime the sequencing reactions.

3. RESULTS AND DISCUSSION

Immunological screening of a λ gt11 library constructed from pea leaf mRNA revealed that 7 out of 8000 plaques expressed protein that cross-reacted with the

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Abbreviations: APX, ascorbate peroxidase; CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; IPTG, isopropylthiogalactoside.

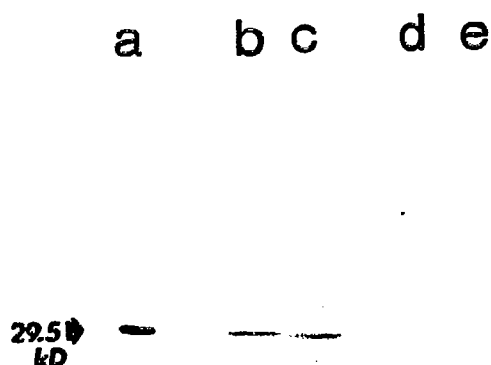


Fig. 1. Western blot analysis of recombinant *E. coli* XL1B cells expressing pea ascorbate peroxidase. (a) Purified pea cytosolic ascorbate peroxidase (1 μ g). (b) Transformed *E. coli* XL1B cells harboring recombinant pBluescript plasmid encoding pea ascorbate peroxidase grown in the absence of IPTG. (c) As in lane b, however grown with 1 mM IPTG. (d) Transformed *E. coli* XL1B cells harboring the non-recombinant pBluescript KS- plasmid. (e) As in lane (d), however induced with IPTG. *E. coli* cells (XL1B) were grown in M-9 minimal medium in the presence of ampicillin, 1 mM IPTG where indicated, and glycerol as a carbon source. Cells (0.4 ml, $OD_{550}=1.2$) were harvested, washed, lysed and boiled in Laemmli sample buffer. SDS-PAGE, electrophoretic transfer of proteins and immunodetection were performed according to [16] and [17], respectively.

antibodies raised against pea cytosolic APX. Two strongly cross-reacting plaques were purified by rescreening at lower plaque density, and phage DNA was subsequently isolated. The cDNA inserts (1 kb) were removed and subcloned into the plasmid vector pBluescript. The expressed product, immunodecorated with the APX-antibody, was of the same M_r as is APX itself, indicating that the insert contained the entire coding sequence (Fig. 1). Expression of the cloned cDNA was independent of the *lac* promoter, as it was affected neither by addition of isopropylthiogalactoside (IPTG) to the medium nor by reversing the orientation of the insert with respect to the *lac* promoter. Therefore, internal to the cDNA 5' flanking sequence is a pseudo-bacterial promoter sequence as well as a potential ribosomal binding site.

The sequencing strategy is shown in Fig. 2. Synthetic primers corresponding to the plasmid vector and internal sites in APX cDNA (as they were determined) were used to prime the sequencing reactions. Both strands were sequenced with no ambiguities. The nucleotide sequence and deduced amino acid sequence of the cDNA insert are shown in Fig. 3. The cDNA contains an ORF of 753 nucleotides, encoding a protein of 27 275

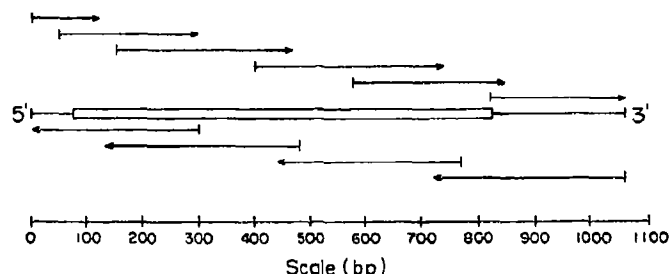


Fig. 2. Sequencing strategy of the cloned cDNA insert. The bar denotes the coding region; the single line indicates the noncoding regions; arrows show the direction and distance of the sequence obtained from each primer.

M_r , in reasonable agreement with a M_r of 29 500 as determined by SDS-PAGE. A putative polyadenylation site (AATAAA) appears 41 bases upstream from the start of the poly(A) tail. The deduced amino acid sequence at the 5' N-terminal region of the cloned cDNA coincides perfectly with the first 33 amino acids obtained by automated Edman degradation of the purified APX from pea [6]. These results indicate that the cloned cDNA does not harbor a transit peptide and is additional support for the cytosolic location of the isozyme.

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GAATTCGGCTTGTGCTCTCTCTCGTGTCACTAGGCTTTAACTTCTTCTGTTTGTCTCTTA
10                               30          50
GATTCGAGAATCGTTTGTCTATGGGAAATCTTACCAACTGTTAGTCCCGATTACGAGA
70                               90          110
AIEKAKRKRLRGFI AEKKCAP
AGGCCATTGAAAGGCTAAGAGGAAGCTCAGAGGTTTATCGCTGAGAAGAAATGCGCTC
130                               150          170
LILRLAWHSAGTTFDSKTKTG
CTCTAATTCCTCGTGGCATGGCAGCTCTGCTGCTACTTTTGATCCAAAGCAAGACTG
190                               210          230
GPFGTIKHQAE LAHGANNGL
GTGGTCTTTTCGGAACAATTAAGCACCAAGCTGAGCTTGCTCATGGTGCTAACAACGGTC
250                               270          290
DIAVRLLEPIKEQFP IVSYA
TTGATATCGCGTTAGGCTGTGGAGGCTATTAGGAGCAATTCCTATTGTGAGCTATG
310                               330          350
DFYQLAGVVAVEITGGGP EVFP
CTGATTTCTACCAAGTTGGCTGGTGTGCTGTGAGATTACCGGTGGACCTGAAGTTC
370                               390          410
FHPGRREDKPEPPPEGRRLPDA
CTTCCACCCTGGTAGGACACAGCCTGAGCCACCCTGAGGCTCGGTTGCCTGATG
430                               450          470
TKGSDHLRDLVFGKAMGLSVV
CCACTAAGGGTTCTGACCATTTGAGGGATGTGTTGGAAAGGCTATGGGGCTTAGTGTAG
490                               510          530
QDIVALS GGH TIGAAH KERS
TACAGGACATTGTTGCTCTATCTGGTGCACACCATTTGAGCTGCACAGCAAGGACGTT
550                               570          590
GFEGPWTSNPLIFDN SYFT E
CTGGATTTGAGGACCATGGACTTCTAATCCTCTCATTTTGACAACCTCATATTTCACTG
610                               630          650
LLTGEKDG L LQLPSDKALLT
AGTTGTTGACTGGTGAGAAGGATGGCCTTCTCAGTTGCCAAGTGATAAGGCACTTTTGA
670                               690          710
DSVFRPLVEKYAAD EDVFFA
CTGACTCTGTATCCGCCCTCTTGTGAGAATATGCTGCGGATGAAGATGTTTCTTTG
730                               750          770
DYAE A H L K L S E L G F A E A *
CTGATTATGCTGAAGCACATCTTAAGTCTCTGAGCTTGGATTGCTGAAGCGTAAGTCA
790                               810          830
CAGTTGTTTGGTGTGTTAGAGAGGAGCACTGTCTGTAATCTTACATAAAATTCATAGCGT
850                               870          890
TGCTTTTATTTTCAATGTGATTCATCTTAGTTGGGTAGCATTTTGGATGTATTTTGAAG
910                               930          950
TTTGATTGTTTCTCTAATTTGTTGATCCTTGGTTAATAACATTGTTAAGTGGTAATGCC
970                               990          1010
AGCTATTGCATTTTTCCTGATAAAAAAACCAGATT
1030                               1050

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Fig. 3. Nucleotide and deduced amino acid sequence of the cDNA encoding pea cytosolic ascorbate peroxidase. The putative polyadenylation signal and possible bacterial promoter and ribosomal binding sites are noted in bold.

Fig. 4. (a) Comparison of the deduced amino acid sequence of pea cytosolic ascorbate peroxidase with sequences of proximal and distal histidine regions of plant, yeast and bacterial peroxidases. HRP C2, horseradish peroxidase C2; TUN P7, turnip peroxidase 7; TOB PER, tobacco peroxidase; POT PER, potato peroxidase; YEA CCP, yeast cytochrome *c* peroxidase; ECO HPI, *E. coli* catalase-peroxidase HPI; BAC PER, *Bacillus stearothermophilus* peroxidase. Sequences were adopted from [18]. Amino acids which are identical to pea ascorbate peroxidase are shaded. Asterisks indicate proximal and distal histidines and the conserved arginine. (b) Comparison of the deduced N-terminal amino acid sequence of pea ascorbate peroxidase with the N-terminal amino acid sequences of chloroplastic ascorbate peroxidases from spinach (SPI APX) [21] and tea (TEA APX) [5].

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- [1] Asada, K. and Takahashi, M. (1987) in: Photoinhibition (Kyle, D.J., Osmond, C.B. and Arntzen, C.J. eds) pp. 227-287, Elsevier, Amsterdam.
- [2] Foyer, C.H. and Halliwell, B. (1976) *Planta* 133, 239-244.
- [3] Shigeoka, S., Nakano, Y. and Kitaoka, S. (1980) *Arch. Biochem. Biophys.* 201, 121-127.
- [4] Dalton, D.A., Russel, S.A., Hanus, F.J., Pascoe, G.A. and Evans, H.J. (1987) *Plant Physiol.* 83, 789-794.
- [5] Chen, G.X. and Asada, K. (1989) *Plant Cell Physiol.* 30, 987-998.
- [6] Mittler, R. and Zilinskas, B.A. (1991) *Plant Physiol.*, submitted for publication.
- [7] Mehlhorn, H., Cottam, D.A., Lucas, P.W. and Wellburn, A.R. (1987) *Free Rad. Res. Comm.* 3, 193-197.
- [8] Tanaka, K., Masuda, R., Sugimoto, T., Omasa, K. and Sakaki, T. (1990) *Agric. Biol. Chem.* 54, 2629-2634.
- [9] Ganitt, J.S. and Key, J.L. (1986) *Mol. Gen. Genet.* 202, 186-193.
- [10] Snyder, M., Elledge, S., Sweetser, D., Young, R.A. and Davis, R.W. (1987) *Meth. Enzymol.* 154, 107-128.
- [11] Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) *Methods in Molecular Biology*, pp. 158-160. Elsevier, New York.
- [12] Hanahan, D. (1985) in: *DNA Cloning: A Practical Approach* (Glover, D.M. ed) Vol. 1, pp. 109-135. IRL, Oxford.
- [13] Birnboim, C. and Doly, J. (1979) *Nucl. Acids Res.* 7, 1513-1523.
- [14] Sanger, F., Nicklen, S. and Coulson, R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
- [15] Hattori, M. and Sakaki, Y. (1986) *Anal. Biochem.* 152, 232-238.
- [16] Laemmli, U.K. (1970) *Nature* 227, 680-685.
- [17] Burnette, W.M. (1981) *Anal. Biochem.* 112, 195-203.
- [18] Fujiyama, K., Takemura, H., Shinmyo, A., Okada, H. and Tanaka, M. (1990) *Gene* 89, 163-169.
- [19] Finzel, B.C., Poulos, T.L. and Kraut, J. (1984) *J. Biol. Chem.* 259, 13027-13036.
- [20] Fishel, L.A., Villafranca, J.E., Mauro, M. and Kraut, J. (1987) *Biochemistry* 26, 351-360.
- [21] Tanaka, K., Takeuchi, E., Kubo, A., Sakaki, T., Kazutomo, H. and Kawamura, Y. (1991) *Arch. Biochem. Biophys.* 286, 371-375.