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

ARTICLE *in* FEBS LETTERS · OCTOBER 1991

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(91)81083-K · Source: PubMed

2 AUTHORS:

Ron Mittler
University of North Texas
5 PUBLICATIONS **1,040** CITATIONS
SEE PROFILE

B. A. Zilinskas
Rutgers, The State University of New Jersey
64 PUBLICATIONS **3,246** CITATIONS
SEE PROFILE

Volume 289, number 2, 257–259 FEBS 10146 September 1991

ADONIS 001457939100845H

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

Ron Mittler and Barbara A. Zilinskas

Department of Biochemistry and Microbiology, Cook College, Rutgers University, New Brunswick, NJ 08903-0231, USA

Received 10 June 1991

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:

2 ascorbate + H_2O_2 → 2 monodehydroascorbate + $2H_2O$.

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.

Correspondence address: B.A. Zilinskas, Department of Biochemistry and Microbiology, Cook College, Lipman Hall, Rutgers University, New Brunswick, NJ 08903-0231, USA. Fax: (1) (908) 932 8965.

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

2. MATERIALS AND METHODS

2.1. *Screening of the λgt11 library*

A cDNA library, constructed from pea leaf mRNA [9] in the expression vector λgt11, 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 λgt11 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 [α-^{35}S]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 λgt11 library constructed from pea leaf mRNA revealed that 7 out of 8000 plaques expressed protein that cross-reacted with the

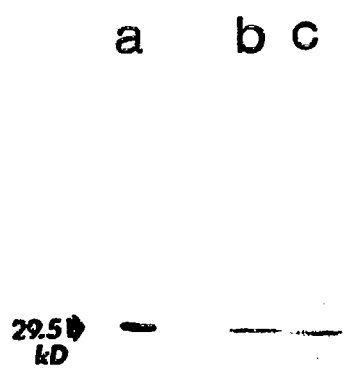

Fig. 1. Western blot analysis of recombinant *E.coli* XL1B cells expressing pea ascorbate peroxidase. (a) Purified pea cytosolic ascorbate peroxidase (1 μg). (b) Transformed *E.coli* XL1B cells harboring recombinant pBluescript plasmid encoding pea ascorbate peroxidase grown in the absence of IPTG. (c) 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

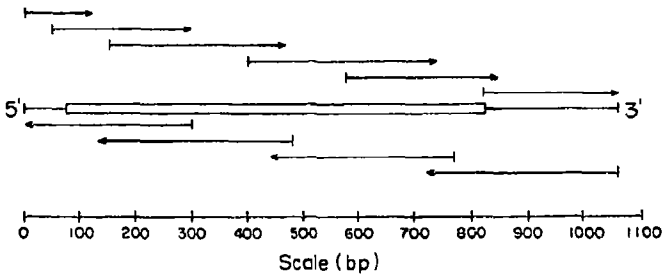

Fig. 2. Sequencing strategy of the cloned cDNA insert. The bar denotes the coding region; the single line indicates the noncoding regions; arrows show the direction and distance of the sequence obtained from each primer.

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.

```
GAATTCGGCTTGTGCTCTCCTCGTGTCACTAGGGTTTAACTTCTTCGTTTTTGCTTCTTA
        10                  30                  50
                  M  G  K  S  Y  P  T  V  S  P  D  Y  Q  K
GATTTCGAGAATCGTTTGCTATGGGAAAATCTTACCCAACTGTTAGTCCCGATTACCAGA
        70                  90                  110
  A  I  E  K  A  K  R  K  L  R  G  F  I  A  E  K  K  C  A  P
AGGCCATTGAAAAGGCTAAGAGGAAGCTCAGAGGTTTTATCGCTGAGAAGAAATGCGCTC
        130                 150                 170
  L  I  L  R  L  A  W  H  S  A  G  T  F  D  S  K  T  K  T  G
CTCTAATTCTCCGTTTGGCATGGCACTCTGCTGGTACTTTTGATTCCAAGACAAAGACTG
        190                 210                 230
  G  P  F  G  T  I  K  H  Q  A  E  L  A  H  G  A  N  N  G  L
GTGGTCCTTTCGGAACAATTAAGCACCAAGCTGAGCTTGCTCATGGTGCTAACAACGGTC
        250                 270                 290
  D  I  A  V  R  L  L  E  P  I  K  E  Q  F  P  I  V  S  Y  A
TTGATATCGCGGTTAGGCTGTTGGAGCCTATTAAGGAGCAATTCCCTATTGTGAGCTATG
        310                 330                 350
  D  F  Y  Q  L  A  G  V  V  A  V  E  I  T  G  G  P  E  V  P
CTGATTTCTACCAGTTGGCTGGTGTTGTTGCTGTTGAGATTACCGGTGGACCTGAAGTTC
        370                 390                 410
  F  H  P  G  R  E  D  K  P  E  P  P  P  E  G  R  L  P  D  A
CTTCCACCCTGGTAGGCGAGACAAGCCTGAGCCACCACCTGAGGGTCGCTTGCCTGATG
        430                 450                 470
  T  K  G  S  D  H  L  R  D  V  F  G  K  A  M  G  L  S  V  V
CCACTAAGGGTTCTGACCATTTGAGGGATGTGTTTGGAAAGGCTATGGGGCTTAGTGTAG
        490                 510                 530
  Q  D  I  V  A  L  S  G  G  H  T  I  G  A  A  H  K  E  R  S
TACAGGACATTGTTGCTCTATCTGGTGGTCACACCATTGGAGCTGCACACAAGGAGCGTT
        550                 570                 590
  G  F  E  G  P  W  T  S  N  P  L  I  F  D  N  S  Y  F  T  E
CTGGATTTGAGGGACCATGGACTTCTAATCCTCTCATTTTTGACAACTCATATTTCACTG
        610                 630                 650
  L  L  T  G  E  K  D  G  L  L  Q  L  P  S  D  K  A  L  L  T
AGTTGTTGACTGGTGAGAAGGATGGCCTTCTTCAGTTGCCAAGTGATAAGGCACTTTTGA
        670                 690                 710
  D  S  V  F  R  P  L  V  E  K  Y  A  A  D  E  D  V  F  F  A
CTGACTCTGTATTCCGCCCTCTTGTTGAGAAATATGCTGCGGATGAAGATGTTTTCTTTG
        730                 750                 770
  D  Y  A  E  A  H  L  K  L  S  E  L  G  F  A  E  A  *
CTGATTATGCTGAAGCACATCTTAAGCTCTCTGAGCTTGGATTTGCTGAAGCCTAAGTCA
        790                 810                 830

CAGTTGTTTGGTGTTTAGAGAGGAGCACTGTCCTGAATCTTACATAAAATTTCATAGACGT
        850                 870                 890

TGCTTTTATTTTCAATGTGATTCATCTTAGTTGGGTAGCATTTTGGATGTATTTTGGAAG
        910                 930                 950

TTTGATTGTTTTCTCTATTGTTGATCCTTGGTTAAATAACATTGTTAAGTGGTAATGCCC
        970                 990                 1010

AGCTATTGCATTTTCCTGATAAAAAAAAAACCGAATT
        1030                1050
```

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.

a.

```
                          Distal His                    Proximal His
                        *   *                               *
PEA APX 31KCAPLILRLAWHSAGTFDSKTKTGGP 149MGLSVVQDIVALSGGHTIGAAHKE
HRP C2  31RIAASILRLFFHDCFVNGCDASILLD 155VGLDRPSDLVALSGGHTFGKNQCR
TUN P7  31RMGASILRLFFHDCFVNGCDGSILLD 155VGLS-TRDMVALSGAHTIGQSRCV
TOB PER 31RAGAKLIRLHFHDCFVNGCDGSILLD 153KGMD-LTDLVALSGAHTFGRARCQ
POT PER 86RMGASLIRLHFHDCFVDGCDGGILLD 211DKNFTLREMVALAGAHTVGFARCS

PEA APX 31KCAPLILRLAWHSAGTFDSKTKTGGP 149MGLSVVQDIVALSGGHTIGAAHKE
YEA CCP 41GVGPVLVRLAWHTSGTWDKHDNTGGS 160QRLNMDREVVALMGAHALGKTHLK
ECO HPI 96SVAGLFIRAMWHGAGTYRSIDGRGGA 255MGMNDEETVALIAGGHTLGKTHGA
BAC PER 90HVGPLFIRMAWHSAGTYRIGDGRGGA 247MGMNDEETVALIAGGHTFGKAHRG
```

b.

```
PEA APX   GKSVPTVSPDVQKAIEK
SPI APX   G-SVPTVHENVQKSIEK
TEA APX   FASDPDELKSAREDIKE
```

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].

Sequence homologies to pea APX were searched in the EMBL data bank; a 33% identity over a 235 amino acid overlap was found with yeast cytochrome *c* peroxidase (CCP), while even lesser homology was found with bacterial peroxidases and classical plant peroxidases. All known peroxidases share three conserved amino acids, namely, His^{52}, His^{175} and Arg^{48} of mature CCP [18]; these amino acids are known to be part of the CCP active site as determined by its solved crystal structure [19]. The deduced amino acid sequence of APX was also found to share the same amino acids (Fig. 4a), and it is suggested that these APX homologous residues participate in charge stabilization (Arg^{38}, and distal His^{42}) and as a heme axil ligand (proximal His^{164}). Yeast and bacterial peroxidases possess a Trp^{41} residue rather than the conserved Phe^{41} seen in all previously described plant peroxidases. Altering CCP Trp^{51} to Phe resulted in changes in its catalytical properties, such that it is more similar to HRP than CCP [20]. In contrast to classical plant peroxidases, APX also possesses Trp^{41} rather than a Phe^{41}; thus, in this respect, APX is more similar to yeast and bacterial peroxidases than to plant peroxidases. Lastly, we note (Fig. 4b) a high degree of homology (70% identity) when a 16 amino acid overlap at the N-terminus of pea cytosolic APX is compared with the recently published N-terminal amino acid sequence of spinach chloroplastic APX [21], although lesser homology was observed with the N-terminal sequence of tea chloroplastic APX [5].

Acknowledgements: We gratefully acknowledge Dr J.S. Gantt for providing the λgt11 library. This is New Jersey Agricultural Experiment Station Publication D-01905-4-91, supported in part by state funds and by the Cooperative State Research Service, U.S. Department of Agriculture, under Agreement No. 89-3471-4502.

REFERENCES

[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] Gantt, 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 Takano, 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.