Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1239–1243, February 1989
Botany

Primary structure of cucumber (*Cucumis sativus*) ascorbate oxidase deduced from cDNA sequence: Homology with blue copper proteins and tissue-specific expression

(multicopper oxidase/copper binding site/protein evolution)

JUN OHKAWA, NAOSUKE OKADA, ATSUHIKO SHINMYO, AND MITSUO TAKANO

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Suita, Osaka 565, Japan

Communicated by Joseph E. Varner, November 21, 1988

ABSTRACT **cDNA clones for ascorbate oxidase were isolated from a cDNA library made from cucumber (*Cucumis sativus*) fruit mRNA. The library was screened with synthetic oligonucleotides that encode the NH_2-terminal sequence of this enzyme. Nucleotide sequence analysis of the cloned cDNA inserts revealed a 1761-base-pair open reading frame that encoded an NH_2-terminal signal peptide of 33 amino acids and a mature enzyme of 554 amino acids (M_r, 62,258). The amino acid sequence deduced from nucleotide sequence analysis agrees with the NH_2-terminal amino acid sequence of the purified ascorbate oxidase, as determined by microsequencing methods. Cucumber ascorbate oxidase contained four histidine-rich regions with striking sequence homology to the corresponding parts of the other multicopper oxidases such as *Neurospora crassa* laccase and human ceruloplasmin and, to some extent, to a low molecular weight copper protein such as plastocyanin. Moreover, these data further support the hypothesis that the small blue copper proteins and the multicopper oxidases have evolved from the same ancestral gene. By RNA blot hybridization analysis, the mRNA for the ascorbate oxidase was found to be abundant in cucumber fruit tissue while expressed at very low levels in leaf and root tissues.**

Ascorbate oxidase (L-ascorbate:oxygen oxidoreductase, EC 1.10.3.3) belongs to a group of multicopper oxidases. The two other members of this group are *Neurospora crassa* laccase and human ceruloplasmin (1). All of these enzymes possess three spectroscopically different copper centers (2). These centers have previously been classified as type 1 or blue, type 2 or normal, and type 3 or coupled binuclear. Ascorbate oxidase catalyzes the oxidation of ascorbate to 2-dehydroascorbate with the concomitant reduction of molecular oxygen to water. This enzyme has been isolated from plants belonging to the family Cucurbitaceae, such as cucumber (*Cucumis sativus*) and squash (*Cucurbita pepo medullosa*) (3, 4). Physical characterization of these enzymes indicated that all native enzymes contained eight copper ions per dimeric enzyme molecule, representing three type 1, one type 2, and four type 3 copper centers (3, 5). However, it remains to be elucidated how these three types of copper function in the oxidation of ascorbate.

The primary structures of the two other multicopper oxidases have been determined by amino acid sequence analysis (6) or by cDNA cloning (7, 8). All the elucidated multicopper oxidase sequences contain clusters of histidine residues, which might be involved in copper binding (9). No detailed information is available on the other multicopper oxidase, ascorbate oxidase. It would be interesting to compare the amino acid sequence of cucumber ascorbate oxidase to those of the other multicopper oxidases to find out whether any homology exists. Such a comparison may also provide the information needed to help clarify the reaction mechanism of ascorbate oxidase. For this reason, we have cloned and characterized cDNAs encoding cucumber ascorbate oxidase.* Using the cloned cDNAs as probes, the levels of expression of the cucumber ascorbate oxidase genes in several plant tissues were also examined.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

Materials. The cucumber (*C. sativus*) plants were grown at a commercial farm near Osaka University and were harvested 7 days after flowering. The fruits, stems, leaves, and roots were separated, frozen in liquid nitrogen, and stored at −80°C until used.

Amino Acid Composition and Sequence Analyses. Ascorbate oxidase purified to homogeneity from cucumber fruits as described (5) was a gift from N. Kasai (Osaka University). The enzyme protein was hydrolyzed with 4 M methanesulfonic acid at 110°C for 24, 48, and 72 hr under vacuum. Amino acid compositions were determined with an amino acid analyzer (model 835, Hitachi, Tokyo). To identify the NH_2-terminal amino acid sequence of this enzyme, the purified ascorbate oxidase (1 nmol) was desalted by HPLC on a TSK G3000SWXL column (0.8 × 30 cm; Toyo Soda, Tokyo) with 50 mM ammonium bicarbonate (pH 7.8). Amino acid sequence analysis by Edman degradation was performed with a model 470A gas-phase protein sequencer (Applied Biosystems). Phenylthiohydantoin-derivatized amino acids were analyzed by on-line HPLC.

Construction and Screening of the cDNA Library. RNA was prepared from cucumber fruits by homogenization in 6 M guanidine thiocyanate followed by centrifugation over a 5.7 M cesium chloride cushion (10). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose column chromatography and then used as the template for synthesis of cDNA transcripts with an oligo(dT) primer and reverse transcriptase (11). Double-stranded cDNA was cloned in the plasmid pUC19 and used to transform *Escherichia coli* HB101 (12, 13). The oligonucleotide mixtures for cDNA screening were designed from the NH_2-terminal amino acid sequence (25 amino acid residues) of the oxidase determined by protein sequencing. A mixture of the eight possible oligonucleotides (probe 1) consisting of 5′-TCCCAYTTRTARTG-3′ and a mixture of the 32 possible oligonucleotides (probe 2) consisting of 5′-AACATRTAYTCNACRTCCCA-3′ (N = A, T, G, or C; R = G or A; and Y = C or T) were synthesized (14). Probes 1 and 2 represent DNA sequences complementary to all possible coding sequences for two regions of ascorbate oxidase; His^7-Tyr-Lys-Trp-Asp and Trp^{10}-Asp-Val-Glu-Tyr-Met-Phe.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04494).

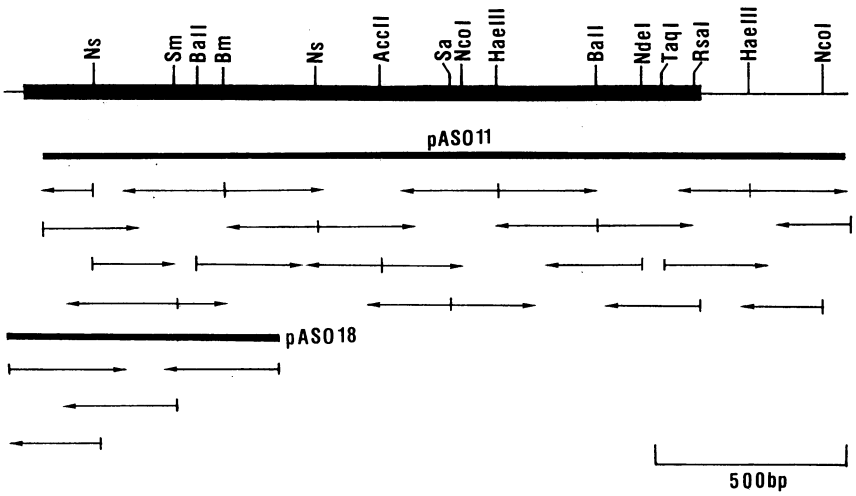

FIG. 1. Restriction map and sequencing strategy of the cucumber ascorbate oxidase (ASO) cDNA clones. Only nonfrequent restriction sites and the sites used for sequence analysis are shown. Arrows indicate direction and extent of sequencing. Solid box in the top line indicates the protein-coding region. Restriction sites are abbreviated as follows: Bm, *Bam*HI; Ns, *Nsp*(7524)I; Sa, *Sau*3AI; Sm, *Sma* I. bp, Base pairs.

Duplicate colony lifts on nitrocellulose filters were hybridized at 33°C for probe 1 and at 46°C for probe 2 (15).

Nucleotide Sequence Analysis. The cDNA inserts of pASO11 and pASO18 were digested by the restriction enzyme and

```
-67                                                                                                    TTTTTTTTTTTTTTTTTTTTTCCTCATTGCATCATTATTCTTCCCTTTCATCTCCTCAACCCTTCGAA   -1

  1 ATGGCAAAAGTTGCAGATAAGCCTTTCTTCCCCAAGCCTTTTCTATCCTTCCTTGTTTTGTCAATCATTTTTGGATTTGGAATAACTCTCTCTGAGGCAGGTTTTCCCAAAATAAAACAC   120
-33 M  A  K  V  A  D  K  P  F  F  F  P  K  P  F  L  S  F  L  V  L  S  I  I  F  G  F  G  I  T  L  S  E  A↓G  F  P  K  I  K  H     7

121 TACAAATGGGATGTTGAGTACATGTTTTGGTCGCCAGATTGTGTTGAAAACATTGTTATGGGAATCAACGGCGAGTTCCCTGGACCGACGATTAGAGCCAACGCTGGCGACATCGTCGTT   240
  8 Y  K  W  D  V  E  Y  M  F  W  S  P  D  C  V  E  N  I  V  M  G  I  N  G  E  F  P  G  P  T  I  R  A  N  A  G  D  I  V  V    47

241 GTGGAGCTAACTAACAAGCTCCACACTGAAGGTGTTGTTATTCATTGGCATGGAATCCTACAACGAGGTACTCCTTGGGCTGATGGCACTGCTTCCATCTCGCAGTGTGCCATTAACCCG   360
 48 V  E  L  T  N  K  L  H  T  E  G  V  V  I  H  W  H  G  I  L  Q  R  G  T  P  W  A  D  G  T  A  S  I  S  Q  C  A  I  N  P    87

361 GGTGAGACCTTCACTTACCGGTTTGTGGTAGATAAGGCTGGGACATATTTCTATCATGGCCATTTAGGGATGCAAAGATCGGCTGGGTTGTATGGATCTTTGATAGTGGATCCACCAGAA   480
 88 G  E  T  F  T  Y  R  F  V  V  D  K  A  G  T  Y  F  Y  H  G  H  L  G  M  Q  R  S  A  G  L  Y  G  S  L  I  V  D  P  P  E   127

481 GGAAGATCTGAGCCATTCCATTATGACGAAGAGATCAACTTATTGCTTAGTGATTGGTGGCATCAGAGTGTTCACAAGCAAGAAGTTGGTCTCAGCTCCAAACCAATGCGTTGGATTGGT   600
128 G  R  S  E  P  F  H  Y  D  E  E  I  N  L  L  L  S  D  W  W  H  Q  S  V  H  K  Q  E  V  G  L  S  S  K  P  M  R  W  I  G   167

601 GAGCCTCAGAGCATATTGATAAATGGGAAAGGGCAATTTGACTGTTCAATAGCAGCCAAATACAACCAAGGTTTGAAGCAATGTGAGTTGAGTGGAAAAGAAAAATGTGCACCATTTATC   720
168 E  P  Q  S  I  L  I  N  G  K  G  Q  F  D  C  S  I  A  A  K  Y  N  Q  G  L  K  Q  C  E  L  S  G  K  E  K  C  A  P  F  I   207

721 CTACATGTTCAACCCAAGAAAACTTATCGGATAAGAATTGCTAGTACCACTGCCTTGGCTTCCCTCAACTTTGCCATTGGAAATCACGAACTGTTAGTGGTAGAAGCCGACGGCAACTAC   840
208 L  H  V  Q  P  K  K  T  Y  R  I  R  I  A  S  T  T  A  L  A  S  L  N  F  A  I  G  N  H  E  L  L  V  V  E  A  D  G  N  Y   247

841 GTTCAACCATTTGTCACTTCCGACATCGACATTTATTCCGGCGAGTCATACTCCGTCCTCATTACCACCGACCAAAACCCATTGGAAAATTACTGGGTATCCATCGGCGTCCGCGCACGG   960
248 V  Q  P  F  V  T  S  D  I  D  I  Y  S  G  E  S  Y  S  V  L  I  T  T  D  Q  N  P  L  E  N  Y  W  V  S  I  G  V  R  A  R   287

961 CTACCCAAAACCCCTCCAGGACTAACCCTCCTCAATTACCTCCCCAACTCCGCCTCCAAATTACCCATTTCTCCACCTCCGGAAACCCCCCACTGGGAGGATTTTGATCGGAGCAAAAAC  1080
288 L  P  K  T  P  P  G  L  T  L  L  N  Y  L  P  N  S  A  S  K  L  P  I  S  P  P  P  E  T  P  H  W  E  D  F  D  R  S  K  N   327

]081 TTCACATTCAGAATCTTCGCTGCCATGGGCAGTCCAAAGCCACCGGTGAGATACAACCGCCGACTCTTCCTCCTCAACACCCAAAATCGAATAAACGGGTTCATGAAATGGGCCATCAAC  1200
328 F  T  F  R  I  F  A  A  M  G  S  P  K  P  P  V  R  Y  N  R  R  L  F  L  L  N  T  Q  N  R  I  N  G  F  M  K  W  A  I  N   367

1201 AATGTCTCTTTAGCTCTCCCTCCAACGCCGTACCTCGCCGCCATGAAAATGAGGCTAAACACTGCCTTCAACCAAAATCCACCACCAGAAACATTCCCATTGAACTACGACATCAACAAC  1320
368 N  V  S  L  A  L  P  P  T  P  Y  L  A  A  M  K  M  R  L  N  T  A  F  N  Q  N  P  P  P  E  T  F  P  L  N  Y  D  I  N  N   407

1321 CCACCGCCGAACCCTGAAACGACAACGGGCAACGGCGTTTACAAGTTCAATATGGGGGAAACGGTAGATGTGATTCTACAAAACGCTAATATGTTAAACCCCAATATGAGTGAGATTCAC  1440
408 P  P  P  N  P  E  T  T  T  G  N  G  V  Y  K  F  N  M  G  E  T  V  D  V  I  L  Q  N  A  N  M  L  N  P  N  M  S  E  I  H   447

1441 CCTTGGCATTTGCATGGCCATGATTTTTGGGTTTTGGGGTATGGAGAGGGGAAATTTTACGCCCCCGAGGATGAGAAGAAACTGAATTTGAAAAATCCACCGTTGAGGAACACAGTGGTG  1560
448 P  W  H  L  H  G  H  D  F  W  V  L  G  Y  G  E  G  K  F  Y  A  P  E  D  E  K  K  L  N  L  K  N  P  P  L  R  N  T  V  V   487

1561 ATTTTCCCATATGGGTGGACGGCGATTCGATTTGTGGCGGATAACCCAGGTGTTTGGGCGTTCCATTGCCATATTGAACCTCATTTGCATATGGGAATGGGAGTTGTGTTTGCCGAAGGA  1680
488 I  F  P  Y  G  W  T  A  I  R  F  V  A  D  N  P  G  V  W  A  F  H  C  H  I  E  P  H  L  H  M  G  M  G  V  V  F  A  E  G   527

1681 GTTCATATGGTGGGAATGATTCCGCCGAAGGCTTTGGCTTGTGGCAGTACCGCGCTGGTTAAGAACTATCCCCGATTACCCTAAAAACCCTAGAAAGAGAAACCCCTAATAAAAAAAACC  1800
528 V  H  M  V  G  M  I  P  P  K  A  L  A  C  G  S  T  A  L  V  K  N  Y  P  R  L  P ***                                        554

1801 CTCGTGAATATTTCAGGAAGGTAAGGTACGTAACCCCTAAAACACCATAGAATGAGAAGCCACTAAAAAACCCTAGGAGGTGGTTTTGGGCCAAGTGGTATATATTCATATTGTGTTTTG  1920
1921 GGGAATATTATAGTTTTGGAGGGTTTAGAGTTGAGGGTGTAGGGAAGTAAAATTAATGTTGTTTAGAGTTAATTTGTTAGTGTGAACTTATCATTGTTATGCTTTTCCTTATATCTTTCT  2040
2041 CTTTTTTTTTGCCATGGTGAAGTGTGCAGGATGAAAAGATTTGTGAAAGGTTTAATTTTGAGGGTTAAAAAAAAAAAAAAAAAAA                                  2125
```

FIG. 2. Nucleotide sequence of the cucumber ascorbate oxidase cDNA. The nucleotide sequence was determined with clones pASO11(+19 to +2125) and pASO18 (−67 to +705). The deduced amino acid sequence (single-letter code) is given under each codon. Nucleotides and amino acids are numbered on both sides. The putative signal peptide cleavage site is shown by a solid arrow. Underlined amino acid sequence is identical to the NH_2-terminal amino acid sequence, which was determined from the purified ascorbate oxidase. The predicted polyadenylylation/processing signal is boxed.

subcloned into M13mp18 and M13mp19 and then subjected to sequence determinations by the chain-termination method (16).

RNA Blot Hybridization Analysis. RNA (20 μg) was denatured with glyoxal and dimethyl sulfoxide, electrophoresed on a 1.1% agarose gel, and then transferred to a nylon membrane (17). The conditions for the latter analysis were as follows: hybridization was carried out at 42°C for 16 hr in a solution containing 50% formamide, 50 mM sodium phosphate buffer (pH 7.0), 0.9 M sodium chloride, 90 mM sodium citrate, 0.02% bovine serum albumin, 0.02% Ficoll, 250 μg of sonicated calf thymus denatured DNA per ml, 0.02% polyvinylpyrrolidone, and ^{32}P-labeled *Eco*RI fragment of pASO11 (specific activity, 4×10^8 cpm/μg) (18). The membrane was then washed with 150 mM sodium chloride containing 15 mM sodium citrate at 42°C for 15 min (four times).

Sequence Comparison. The amino acid sequence deduced from the cDNA sequence was subjected to Protein Research Foundation data base. For sequence comparison, conservative amino acid substitutions were defined as residues belonging to one of the following six groups: Cys; Ser, Thr, Pro, Ala, Gly; Asn, Gln, Asp, Glu; His, Arg, Lys; Met, Ile, Leu, Val; Phe, Tyr, Trp (19).

RESULTS AND DISCUSSION

Isolation and Nucleotide Sequence Analysis of Cucumber Ascorbate Oxidase cDNA. Cucumber ascorbate oxidase purified to homogeneity was shown by NaDodSO$_4$/polyacrylamide gel electrophoresis analysis to consist of a single component with an M_r of 68,000. This enzyme gave the following amino acid sequence from the NH_2 terminus: Gly-Phe-Pro-Lys-Ile-Lys-His-Tyr-Lys-Trp-Asp-Val-Glu-Tyr-Met-Phe-Trp-Ser-Pro-Asp-Xaa-Val-Glu-Asn-Ile. From this sequence, we designed two mixtures of synthetic oligonucleotides (probes 1 and 2) to represent all possible coding sequences for two portions of ascorbate oxidase amino acid sequences as described in *Experimental Procedures*.

About 85,000 independent transformants from a cucumber cDNA library were first screened with the ^{32}P-labeled probe 2. Six clones that were identified that hybridized with probe 2 were also found to hybridize to probe 1. Individual clones were designated pASO11, -14, -18, -20, -191, and -192, and were subjected to further analysis. Preliminary sequence analysis showed pASO11 contained the longest cDNA insert but lacked the 5′-terminal region of the coding sequence. Restriction endonuclease mapping of the other clones demonstrated that five clones contained a similar size cDNA insert and belonged to a single group (data not shown), probably derived from the same mRNA (Fig. 1). pASO18 was shown to contain a partial ascorbate oxidase mRNA sequence encoding the 5′-noncoding region and the NH_2-terminal portion of the enzyme. This result is explained by assuming that the oligo(dT) nucleotide may have hybridized to adenine-rich sequences in the mRNA (might be 5′-AAAAGAAAAA-3′ at 696 nucleotides downstream from the initiation ATG) and served to prime cDNA synthesis by reverse transcriptase. Two clones, pASO11 and pASO18, were then sequenced (Fig. 2). No sequence difference was observed in the overlapping region on these two clones.

Analysis of the complete nucleotide sequences of the cloned cDNAs revealed one open reading frame of 587 amino acid residues encoding ascorbate oxidase. This sequence also included an NH_2-terminal extension of 33 amino acid residues that is removed prior to the appearance of mature ascorbate oxidase. This result was from the following observations: (*i*) the NH_2-terminal amino acid sequence of ascorbate oxidase, as determined by sequential Edman degradation analysis, is identical to that deduced from cDNA sequence analysis, and (*ii*) the nucleotide sequence around the first ATG triplet agrees well with the favored sequence that flanks functional codons of $\overset{A}{G}$NNATGG (where N = any nucleotide) (20). The open reading frame is followed by a 3′ untranslated region of 406 base pairs and a poly(A) track. Other reading frames were interrupted by multitermination codons. The 3′ untranslated region contained the common polyadenylylation/processing signal, 5′-AATAAA-3′ located 315 nucleotides upstream from the poly(A) track.

Primary Structure of Ascorbate Oxidase. The primary structure of cucumber ascorbate oxidase deduced from the cDNA nucleotide sequence is shown in Fig. 2. The NH_2-terminal amino acid sequence determined by protein sequencing (Gly-1 to Ile-25) is identical with that deduced from cDNA sequencing, suggesting the presence of a signal peptide at the NH_2 terminus. Mature ascorbate oxidase was deduced to contain 554 amino acid residues, which corresponds to a calculated M_r of 62,258. Since the subunit M_r of this enzyme has been shown by NaDodSO$_4$/polyacrylamide gel electrophoresis to be ≈68,000, we suspect that cucumber ascorbate oxidase is also glycosylated as in squash. Esaka *et al.* (21) have shown that cultured squash (*Cucurbita* sp. Ebisu Nankin) cells excrete mature ascorbate oxidase as a glycoprotein into the medium. The carbohydrate content of this enzyme was determined to be 10% (4). Cucumber ascorbate oxidase has three potential N-glycosylation sites (Asn-Xaa-Ser/Thr) at residues 327, 368, and 442. If cucumber ascorbate oxidase is glycosylated at these sites to the same extent as squash ascorbate oxidase, the calculated size of this enzyme would be in close agreement with its size based on electrophoretic mobility. Furthermore, the amino acid composition of the enzyme as deduced from the cDNA sequence agrees reasonably well with that experimentally determined for the purified enzyme from cucumber fruits (Table 1).

It has been suggested that cucumber ascorbate oxidase is synthesized as a prepeptide containing an NH_2-terminal signal peptide of 33 amino acid residues, which is removed by posttranslational processing upon export. The signal peptide with a putative initiator methionine is rich in hydrophobic amino acid residues and might function in the initiation of export of nascent polypeptide chains across the rough endoplasmic reticulum (22, 23). The cDNA sequence predicts that

Table 1. Comparison of the amino acid composition (expressed as residues per subunit) of purified enzyme and that derived from the cDNA sequence of cucumber ascorbate oxidase

Amino acid	Predicted from DNA sequence	Analysis of purified ascorbate oxidase
Lysine	27	26.6
Histidine	21	20.5
Arginine	20	19.1
Asparagine	40	59.9
Aspartate	20	
Threonine	29	28.6
Serine	28	26.6
Glutamine	15	44.3
Glutamate	29	
Proline	50	50.1
Glycine	43	43.2
Alanine	33	32.8
Cysteine	7	5.5
Valine	36	35.5
Methionine	15	14.9
Isoleucine	34	32.7
Leucine	45	44.6
Tyrosine	22	21.7
Phenylalanine	26	25.8
Tryptophan	14	13.9

```
A     Lac 31   NTGKTRRYKLTLTETDNWLGPDGVIKDKVMMVNDNIIGPTIQADWGDYIEITVINKL
                 ** ****  * *   * *** *  * ** ** *  **** ** ** * * * ***
      Aso  1   GFPKIKHYKWDV-EYMFW-SPDCV-ENIVMGINGEFPGPTIRANAGDIVVVELTNKL

      Lac 87   KSNGTSIHWHGMHQRNSNIQDGVNGVTECPIPPRGGSKVYRWPATQYGTSWYHSHFS
               ****  ****** ** *   **  ******* * * *  ***     ** ***** *
      Aso 55   HTEGVVIHWHGILQRGTPWADGTASISQCAINP-GETFTYRFVVDKAGTYFYHGHLG

B     Lac428   HPIHLHGHDFLILGRSPDVTAISQTRYVFDPAVDMARLNGNNPTRRDTAML-PAKGW
               ** ******* ***         **** * *  *   **  **  ***  * *  **
      Aso447   HPWHLHGHDFWVLG-----Y-G-EGKF-YAPEDEKK-LNLKNPPLRNTVVIFP-YGW

      Lac484   LLIAFRTDNPGSWLMHCHIAWHVSGGLSNQFLE
                 * *  **** *  ****  *   * *  * *
      Aso494   TAIRFVADNPGVWAFHCHIEPHLHMGMGVVFAE
```

FIG. 3. Amino acid sequence (single-letter code) comparison between NH_2- and COOH-terminal parts (*A* and *B*) of cucumber ascorbate oxidase and homologous regions of laccase. Lac, *N. crassa* laccase; Aso, cucumber ascorbate oxidase. Asterisks between the sequences indicate identity or conservative substitutions of the two sequences. Numbers to the left refer to the amino acid residue locations with the NH_2-terminal amino acid residue (Gly) of the purified ascorbate oxidase designated as +1.

an Ala-Gly bond is cleaved during the removal of the signal peptide. This is consistent with demonstrated signal peptidase cleavage specificity (24–26).

Sequence Comparison of Cucumber Ascorbate Oxidase with Other Blue Copper Proteins. The amino acid sequences of plastocyanins with single type 1 copper ion from a variety of sources have been determined (27). X-ray crystallography of the poplar plastocyanin revealed a three-dimensional structure as well as amino acid residues that are involved in copper binding (28). The primary structure of the multicopper oxidase, such as human ceruloplasmin (6) and *N. crassa* laccase (8), was recently determined by amino acid sequencing and cDNA analysis. The amino acid sequence of the COOH-terminal portion of these multicopper oxidases had homology with that of the plastocyanin (9). Previous studies suggest that the multicopper oxidase and small blue copper proteins such as plastocyanins were derived from the same ancestral gene. This hypothesis also seems to apply to the cucumber ascorbate oxidase.

Fig. 3 shows comparison of the amino acid sequence of the NH_2- and COOH-terminal portions of cucumber ascorbate oxidase (Gly-1 to Gly-110 and His-447 to Glu-526) with those of *N. crassa* laccase (Glu-31 to Ser-143 and His-428 to Glu-516). The degree of sequence identity between homologous portions of ascorbate oxidase and laccase at NH_2 and COOH termini are 38% and 46%. The homology increases to 64% and 59% if conservative amino acid substitutions are included. However, comparison of internal portions of both enzymes shows lower sequence homology, and the whole amino acid sequence of cucumber ascorbate oxidase has sequence identity (30%) with that of *N. crassa* laccase. Messerschmidt *et al.* (29, 30) recently determined the three-dimensional structure and copper-binding site of ascorbate oxidase prepared from zucchini. Zucchini ascorbate oxidase exhibited striking sequence identity (≈90%) to cucumber ascorbate oxidase. On the basis of this crystallographic analysis, it could be proposed that each subunit has four copper ions, such as one type 1, one type 2, and two type 3 copper centers. All of the copper binding amino acid residues in zucchini ascorbate oxidase deduced from the three-dimensional model are identical at the corresponding positions of cucumber ascorbate oxidase: His-447, Cys-510, His-515, and Met-520 as type 1; His-62 and His-450 as type 2; and His-64, His-106, His-108, His-452, His-509, and His-511 as type 3 copper ligands. These amino acid residues were located in four short stretches with extensive sequence homology among multicopper oxidases (Fig. 4). The amino acid sequences of the NH_2-terminal portion, including sequences in Fig. 4 *A* and *B*, and the COOH-terminal portion, including sequences in Fig. 4 *C* and *D*, of cucumber ascorbate

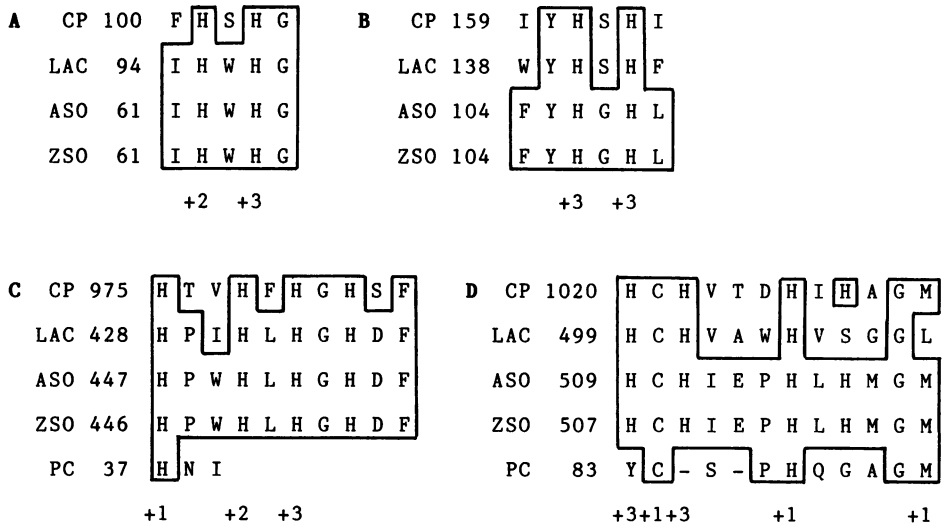

FIG. 4. Amino acid sequence (single-letter code) comparison of four highly conserved regions (*A*, *B*, *C*, and *D*) of cucumber ascorbate oxidase (ASO), zucchini ascorbate oxidase (ZSO), *N. crassa* laccase (LAC), human ceruloplasmin (CP), and poplar plastocyanin (PC). Numbers on the left of each sequence identify the positions within the proteins of the first residues shown. Only amino acid residues identical for ascorbate oxidase are boxed. Potential ligands to the three different types of copper are indicated by +1, +2, +3, respectively. The assignment of the copper ligands for laccase and ceruloplasmin is based on that proposed for zucchini ascorbate oxidase (30).

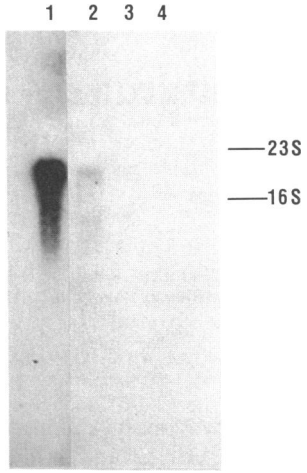

FIG. 5. RNA blot analysis of total RNA from various cucumber tissues probed with a cDNA clone to cucumber ascorbate oxidase. Twenty micrograms of total RNA isolated from cucumber fruit (lane 1), stem (lane 2), leaf (lane 3), and root (lane 4) was applied to a 1.1% agarose gel. Mobilities of the *E. coli* 23S (2.9 kilobases) and 16S (1.5 kilobases) rRNA are indicated. The hybridization signal of mRNA prepared from cucumber leaf tissue in lane 3 is not strong enough to photographically reproduce.

oxidase exhibited sequence similarity to those of the corresponding portions of *N. crassa* laccase without insertion of more than six amino acid residues. This sequence similarity also strongly supported the notion that these four regions may play an important role in the formation of the active site of multicopper oxidase. Furthermore, the copper binding amino acid residues of the plastocyanin elucidated from x-ray studies are completely conserved at the corresponding portions of ascorbate oxidase (His-447, Cys-510, His-515, and Met-520), but the corresponding portion of ascorbate oxidase (Leu-401 to Val-528) had the lower degree of sequence identity (11%) with the amino acid sequence of the poplar plastocyanin.

Tissue-Specific Expression of Cucumber Ascorbate Oxidase. Total RNA prepared from healthy cucumber leaf, stem, fruit, and root tissues were hybridized to the ascorbate oxidase cDNA clone under stringent conditions. The results shown in Fig. 5 reveal an abundant transcript of ≈2200 nucleotides in fruit and stem tissues and a low abundance in leaf and root tissues. These results agree with the specific enzyme activity of cucumber ascorbate oxidase in each tissue (data not shown). Although one of the possible functions of ascorbate oxidase may be involvement in lignin biosynthesis, a greater abundance of ascorbate oxidase mRNA in cucumber fruit tissue than in stem and leaf tissue could suggest another function of ascorbate oxidase in this tissue.

1. Malmstrom, B. G., Andreasson, L. E. & Reinhammar, B. (1975) in *The Enzymes*, ed. Boyer, P. D. (Academic, Orlando, FL), Vol. 12, pp. 507–579.
2. Malkin, R. & Malmstrom, B. G. (1970) *Adv. Enzymol.* **33,** 177–244.
3. Nakamura, T., Makino, N. & Ogura, Y. (1968) *J. Biochem. (Tokyo)* **64,** 189–195.
4. Stark, G. T. & Dawson, C. R. (1962) *J. Biol. Chem.* **237,** 712–716.
5. Marchesini, A. & Kroneck, P. M. H. (1979) *Eur. J. Biochem.* **101,** 65–76.
6. Takahashi, N., Ortel, L. & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 390–394.
7. Koschinsky, M. L., Funk, W. D., van Oost, B. A. & MacGillivray, R. T. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5086–5090.
8. Germann, U. A., Muller, G., Hunziker, P. E. & Lerch, K. (1988) *J. Biol. Chem.* **263,** 885–896.
9. Germann, U. A. & Lerch, K. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8854–8858.
10. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 196–198.
11. Gubler, U. & Hoffman, B. J. (1983) *Gene* **25,** 263–269.
12. Huynh, T., Young, R. A. & Davis, R. W. (1985) in *DNA Cloning: A Practical Approach,* ed. Glover, D. M. (IRL, Oxford), pp. 49–78.
13. Hanahan, D. (1985) in *DNA Cloning: A Practical Approach,* ed. Glover, D. M. (IRL, Oxford), pp. 109–135.
14. Adams, S. P., Kavka, K. S., Wykes, E. J., Holder, S. B. & Gallupi, G. P. (1983) *J. Am. Chem. Soc.* **105,** 661–663.
15. Nawa, H., Hirose, T., Takashima, H., Inayama, S. & Nakanishi, S. (1983) *Nature (London)* **306,** 32–36.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5205.
18. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
19. Dayhoff, M. O., Schwartz, R. M. & Orcutt, B. C. (1978) in *Atlas of Protein Sequence and Structure*, ed. Dayhoff, M. O. (Natl. Biomed. Res. Found., Silver Spring, MD), Vol. 5, Suppl. 3, pp. 345–352.
20. Kozak, M. (1984) *Nucleic Acids Res.* **12,** 857–872.
21. Esaka, M., Imagi, J., Suzuki, K. & Kubota, K. (1988) *Plant Cell Physiol.* **29,** 231–235.
22. von Heijne, G. (1982) *J. Mol. Biol.* **159,** 537–541.
23. Watson, M. E. E. (1984) *Nucleic Acids Res.* **12,** 5143–5164.
24. von Heijne, G. (1983) *Eur. J. Biochem.* **133,** 17–21.
25. Lagrimini, L. M., Burkhart, W., Moyer, M. & Rothstein, S. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7542–7546.
26. Fujiyama, K., Takemura, H., Shibayama, S., Kobayashi, K., Choi, J.-K., Shinmyo, A., Takano, M., Yamada, Y. & Okada, H. (1988) *Eur. J. Biochem.* **173,** 681–687.
27. Adman, E. T. (1985) in *Metalloproteins,* ed. Harrison, P. M. (Macmillan, London), Part 1, pp. 1–42.
28. Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M. & Venkatappa, M. P. (1978) *Nature (London)* **272,** 319–324.
29. Messerschmidt, A., Rossi, A., Ladenstein, R., Huber, R., Bolognesi, M., Marchesini, A., Petruzzelli, R. & Finazzi-Agro, A. (1988) in *Oxidases and Related Redox Systems*, eds. King, T. K., Mason, H. S. & Morrison, M. (Liss, New York), pp. 285–288.
30. Messerschmidt, A., Rossi, A., Ladenstein, R., Huber, R., Bolognesi, M., Gatti, G., Marchesini, A., Petruzzelli, R. & Finazzi-Agro, A. (1988) *J. Mol. Biol.,* in press.