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Induction of 33-kD and 60-kD Peroxidases during Ethylene-Induced Senescence of Cucumber Cotyledons

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ABSTRACT

Ethylene enhanced the senescence of cucumber (*Cucumis sativus* L. cv 'Poinsett 76') cotyledons. The effect of 10 microliters per liter ethylene was inhibited by 1 millimolar silver thiosulfate, an inhibitor of ethylene action. An increase in proteins with molecular weights of 33 to 30 kilodaltons and lower molecular weights (25, 23, 20, 16, 12, and 10 kilodaltons) were observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels after ethylene enhanced senescence. The measurement of DNase and RNase activity in gels indicated that these new proteins were not nucleases. Two proteins from ethylene-treated cotyledons were purified on the basis of their association with a red chromaphore and subsequently were identified as peroxidases. The molecular weights and isoelectric points (pI) of two of these peroxidases were 33 kilodaltons (cationic, pI = 8.9) and 60 kilodaltons (anionic, pI = 4.0). The observation that [^{35}S]Na_2SO_4 was incorporated into these proteins during ethylene-enhanced senescence suggests that these peroxidases represent newly synthesized proteins. Antibodies to the 33-kilodalton peroxidase precipitated two *in vitro* translation products from RNA isolated from ethylene-treated but not from control cucumber seedlings. This indicates that the increase in 33-kilodalton peroxidase activity represents *de novo* protein synthesis. Both forms of peroxidase degraded chlorophyll *in vitro*, which is consistent with the hypothesis that peroxidases have catabolic or scavenging functions in senescent tissues.

Ethylene is known to accelerate senescence in plants (1). While the loss of RNA, Chl, and protein during senescence is known (17, 22), the enzymology of the degradation process is not understood. The original purpose of the work reported here was to characterize the enzymes involved in nucleic acid degradation. As an experimental system, we chose to measure enzyme changes during ethylene-enhanced senescence in cotyledons of 2-week-old cucumber seedlings since Lewington *et al.* (9) reported that yellowing of cucumber cotyledons was associated with a decrease in RNA, DNA, and protein and with an increase in RNase and DNase activity. While new proteins were detected in SDS-PAGE gels during senescence, none were associated with nuclease activity as measured in SDS-PAGE nuclease activity gels. However, two proteins with red chromaphores, which were subsequently found to be peroxidases, appeared during ethylene-induced senescence. This paper describes the isolation and purification of these enzymes.

In spite of the fact that plant peroxidases have been studied by many workers, their physiological functions are only partially understood (7). One function ascribed to peroxidase is that of a Chl-degrading enzyme (12, 23). However, two other Chl-degrading systems have also been proposed. These are chlorophyllase (16) and Chl oxidase (11). Matile *et al.* (13) have presented evidence that pink bilirubin-like compounds with an absorption maximum at 562 nm may represent the breakdown products of Chl.

Evidence has accumulated that peroxidase-based reactions may play a role in senescence. Lauriere (8) showed that peroxidase activity often increased during senescence. More specifically, Ford and Simon (6) have reported that peroxidase activity increased during senescence of cucumber plants. Three observations that support the idea that peroxidation is a part of senescence are: (a) catalase levels decreased during leaf senescence (8), (b) externally applied H_2O_2 increased leaf senescence (18), and (c) levels of H_2O_2 increased during the related process of fruit ripening (5).

This paper describes the isolation, purification, and synthesis of peroxidases associated with cucumber cotyledon senescence. The ability of cucumber peroxidases to function as Chl-degrading enzymes was also studied.

MATERIALS AND METHODS

Plant Material. Cucumber (*Cucumis sativus* L. cv 'Poinsett 76') seeds were obtained from W. Atlee Burpee Co, Warminster, PA.[1] Plants were grown in the greenhouse in 57 × 27 × 7 cm trays filled with vermiculite and watered with 5 g L^{-1} Peters soluble 20-20-20 fertilizer (W. R. Grace & Co, Allentown, PA).

Cotyledon Senescence System. For the senescence experiments described in Tables I and II, four cotyledons were cut from 2-week-old seedlings and placed on moist Whatman No. 3 filter paper in 9 cm Petri plates. The Petri plates were placed in 4 L plastic containers fitted with rubber vaccine stoppers and were stored in the dark at 30°C. Ethylene was injected into the containers with a syringe. Cotyledons were treated with 1 mM STS[2] and 1 to 100 μM AVG by dipping them in the appropriate solution with 0.05% Triton X-100 as a surfactant and then

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

[2] Abbreviations: STS, silver thiosulfate; AAB, ammonium acetate pH 5 buffer; AVG, aminoethoxyvinylglycine; CNBr, cyanogen bromide; CPO, cucumber peroxidase; 33- and 60-CPO, 33- and 60-kD cucumber peroxidase; NBD, 2,5-norbornadiene; pI, isoelectric point.

placing them on the moist filter paper. Preparation of STS and the method used to measure ethylene has been described earlier (2, 3). At the end of the 3-d incubation period, one-quarter (approximately 50 mg) of the cotyledon was excised, placed in 3 ml of 80% ethanol in water, heated at 80°C 10 min to extract the Chl, and cooled to room temperature, and the optical density of the alcohol extract was measured at 665 nm. Data were calculated on the basis of A_{665} nm per g fresh weight of tissue. Values shown represent the average of eight samples. Each experiment was repeated three times.

Protein Determination. Proteins were measured by means of the Bio-Rad protein assay reagent (Bio-Rad, 2200 Wright Ave., Richmond, CA 94804) using directions supplied by the manufacturer.

Polyacrylamide Electrophoresis. SDS-PAGE in 15% acrylamide was performed as described by Maniatis *et al.* (10). Except where noted in Figure 1, all samples were applied in loading buffer consisting of 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 20% glycerol, and 0.02% bromophenol blue and were heated for 3 min at 100°C. Gels were stained with 0.1% Coomassie brilliant blue R dissolved in 25% methanol, 10% acetic acid in water. Gels were dried on a slab-gel dryer. Autoradiograms were prepared by exposing dried gels to Kodak XAR-5 film at room temperature.

The proteins for lanes 1 through 3 in the SDS-PAGE gel shown in Figure 1 were isolated from acetone powders of cucumber cotyledons. Acetone powders were prepared at room temperature by homogenizing 10 cotyledons (about 2 g fresh weight) in 12 ml 70% (v/v) acetone with a Brinkman Kinematica homogenizer. The homogenate was washed on a Büchner funnel with 12 ml 70% acetone and three 10 ml washes of 100% acetone. Proteins from the acetone powder (about 100 mg) were extracted with 2 ml 0.1 M (pH 6.8), KPi buffer. The powder was mixed with the buffer on a Vortex shaker, placed under vacuum to remove entrapped air, and centrifuged at 20,000*g* for 10 min. The resulting 1 ml supernatant (about 1 mg/ml protein) was mixed with loading buffer and heated for 3 min at 100°C. After cooling, precipitates were removed by centrifugation for 2 min in an Eppendorf centrifuge. Each lane was loaded with 50 μl supernatant containing about 30 μg protein. Lanes 4 through 7 were loaded with 2 μg protein.

Isoelectric focusing gels were run in Bio-Lyte 3/10 ampholytes and stained according to directions supplied by Bio-Rad. Peroxidase activity was measured by washing gels twice for 10 min in 0.1 M Tris (pH 7) buffer, followed by a 5 min incubation in 0.1 M Tris (pH 7), 2.9 mM H_2O_2, 13.4 mM guaiacol. Gels were washed with water and dried as described above.

RNase and DNase Activity Gels. Localization of nuclease activity was accomplished by the method of Blank *et al.* (4). Gels were incubated for 18 h before staining with toluidine blue.

Treatment of Cucumber Seedlings with Ethylene. Two-week-old cucumber seedlings were treated with 100 μl/L ethylene for 3 to 6 d in 40-L Plexiglas (62 × 32 × 24 cm) boxes at room temperature and constant fluorescent room illumination. The boxes were opened daily, vented, and reinjected with 4 ml of ethylene. For some experiments, seedlings were sprayed to runoff with 20 mM 2-chloroethylphosphonic acid (ethephon) in 0.05% Triton X-100 and were harvested after 3 d. Controls were sprayed with 0.05% Triton X-100.

Peroxidase Purification. Ethylene-treated seedlings (250 g) were collected and homogenized at room temperature for 1 min with 400 ml of 20 mM sodium acetate (pH 5) in a Waring blender operated full speed. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 5,000*g* for 15 min. Proteins were precipitated by mixing 500 ml of the homogenate with 1.5 L ethanol and centrifuging the mixture at 1,500*g* for 15 min. The alcohol was discarded, and the pellet was resuspended in 80 ml water. The suspension was then dialyzed at 4°C for 40 h with three changes of water. The solution was clarified by centrifugation at 5,000*g* for 15 min. Water was removed by lyophilization, yielding about 250 mg of powder containing 25% protein. The powder was then dissolved in 10 ml of 20 mM AAB, centrifuged at 12,000*g* for 10 min to remove insoluble material, and loaded on a 2.5 cm × 13 cm DEAE Bio-Gel A (Bio-Rad Labs) column. Proteins were eluted at a rate of 1.3 ml min^{-1} with a gradient of 200 ml each of AAB and AAB with 0.5 M NaCl. Using A_{400} nm or red color as a guide, two peaks of red protein were collected, the first between 40 and 70 ml and the second between 125 and 150 ml. These two column fractions were dialyzed against two changes of water and lyophilized. Both fractions were purified further by chromatography on a Sephacryl S-200 column (2.4 × 95 cm) eluted with 20 mM AAB at a rate of 1 ml min^{-1}. After calibrating the Sephacryl S-200 column with mol wt standards, the first red protein peak from the DEAE Bio-Gel A column was found to have a mol wt of about 33 kD (33-CPO), and the second, 60 kD (60-CPO). After dialysis and lyophilization, the yield of 33-CPO was 13 mg of protein and of 60-CPO, 2 mg. The 33-CPO gave a single band on SDS-PAGE (see Fig. 1, lane 4) and isoelectric focusing gels. The 60-CPO still had two contaminating proteins. Contaminating proteins from 60-CPO were removed by chromatography on a 2.5 × 13 cm CM Bio-Gel A column. Proteins were eluted at a rate of 1.3 ml min^{-1} with a gradient of 200 ml each of 20 mM sodium acetate (pH 5), and 20 mM sodium acetate with 0.5 M NaCl. The red protein peak near the void volume was collected, dialyzed, and lyophilized. SDS-PAGE of this protein after heating showed one band with two faint adjacent bands in the 30-kD region (Fig. 1, lanes 5 and 7) which is consistent with the interpretation that either the 60-CPO protein consisted of two subunits or heating in SDS markedly altered the shape of the native protein.

HPLC Chromatography. The red chromaphore was separated from 33-CPO by chromatography on a Perkin Elmer HS-5 C18 HPLC column developed with a linear gradient of 10 to 60% acetonitrile in water with 0.1% trifluoroacetic acid. Presence of the chromaphore was determined by monitoring the column effluent at 398 nm. Except for monitoring the column effluent of 200 nm, the same technique was used to separate CNBr fragments of 33-CPO.

[^{35}S]Na_2SO_4 Incorporation into Cotyledon Proteins. Three sets of cotyledons were used in this experiment: initials (cotyledons harvested from untreated plants), 3-d-air, and 3-d-ethylene (cotyledons from plants incubated in gassing chambers with air or 100 μl/L ethylene, respectively for 3 d). Three cotyledons from each treatment were placed on moist filter paper in Petri plates and treated with [^{35}S]Na_2SO_4 (DuPont NEN Research Products, Boston, MA). The [^{35}S]Na_2SO_4 (0.23 mBq in 10 μl) was placed on the upper side of the cotyledon and spread out with a glass rod. The cotyledons were then incubated at 25°C for 24 h 30-cm away from a 40 W fluorescent light, in glass 6-L desiccators. The desiccators were filled with air for initial and 3-d-air cotyledons or with 100 μl/L ethylene for 3-d-ethylene cotyledons. The cotyledons were rinsed with water and disrupted in a glass conical homogenizer with 1 ml 50 mM sodium acetate (pH 5) buffer. A 1 ml portion of the homogenate was then centrifuged in an Eppendorf centrifuge for 1 min. The supernatant (0.9 ml) was added to 3.6 ml of ethanol and centrifuged at 1500*g* for 2 min. The precipitate was washed twice with 1 ml of 80% ethanol at 1500*g* for 2 min and then dissolved in 0.5 ml water. Insoluble material was removed by centrifugation in an Eppendorf centrifuge for 1 min. The supernatant was transferred to an Eppendorf tube and dried in a Savant Speed Vac Concentrator. The pellet was dissolved in water to yield about 2 mg ml^{-1} protein, and 20 μg protein were loaded in a slot for SDS-PAGE. After electro-

phoresis, the gels were stained, dried, and exposed to x-ray film for 7 d.

Amino Acid Analysis. Samples were hydrolyzed in sealed tubes in 6 N HCl under vacuum at 110°C for 24 h. The samples were then dried by roto-evaporation and reconstituted in 0.2 N lithium citrate. The amino acid content was determined using a 121MB Beckman Instruments Inc. amino acid analyzer with a sequence of lithium citrate buffers described by the manufacturer. The amino acid sequence of CNBr fragments was determined by means of an Applied Biosystems 470A gas phase protein sequencer. The protein identification database of the National Biomedical Research Foundation (Georgetown Univ. Med. Center, 3900 Reservoir Road, N.W., Washington, DC 20007) was used to identify CNBr fragments.

Antibody Production. Antibodies to purified 33-CPO were raised in rabbits by Behring Diagnostics (10933 N. Torrey Pines Rd., LaJolla, CA 92037). These antibodies did not cross-react with 60-CPO.

RNA Extraction from Cotyledons. RNA was extracted from lyophilized cotyledons by a modification (our manuscript in preparation) of a procedure described by Mozer (15).

***In Vitro* Translation of RNA.** *In vitro* translation of total cucumber cotyledonary RNA was performed in the rabbit reticulocyte translation system supplied by Bethesda Research Laboratories.

Immunoprecipitation of *in Vitro* Translation Products. Precipitation of *in vitro* translation products was accomplished as described by Sato *et al.* (19).

Neutron Activation Analysis. Metal ion content of the 33-CPO was determined by neutron activation analysis. A 1 mg sample of purified 33-CPO was analyzed for 40 different elements by Nuclear Energy Services (Nuclear Engineering Department, North Carolina State University, Raleigh NC 27695).

Chl Peroxidase Assay. The procedure of Yamauchi and Minamide (23) was used to measure Chl peroxidation. Chl *a* and *b* were supplied by Serva Fine Biochemicals (200 Shames Drive, Westbury NY 11590). Changes in the concentration of Chl *a* and *b* were measured at 673 nm and 650 nm, respectively.

RESULTS AND DISCUSSION

The data in Table I show that ethylene accelerated the loss of Chl in isolated cucumber cotyledons. In agreement with other ethylene-mediated effects such as seed germination (2) and root growth (3), STS retarded the effect of ethylene.

Chl loss observed in the absence of added ethylene may be due to endogenously produced ethylene. Support for this interpretation was obtained (Table II) by showing that AVG, an inhibitor of ethylene production (3), inhibited Chl degradation but that externally supplied ethylene overrode the AVG effect.

Table I. *Effect of Ethylene and 1 mM STS on the Chl Content of Cucumber Cotyledons*

Data shown are means of eight samples ± SD after a 3-d incubation in the dark at 30°C. Identical letters indicate that means are similar at the 5% level.

Treatment	Chl
	A_{665}/g fresh wt
Initial, 0 d	23 ± 2 a
Air	17 ± 3 c
0.1 μl/L ethylene	12 ± 2 d
1.0 μl/L ethylene	11 ± 3 d
10.0 μl/L ethylene	8 ± 2 de
STS	17 ± 3 c
STS + 10.0 μl/L ethylene	18 ± 1 c

Table II. *Effect of AVG on Ethylene Production and Senescence of Cucumber Cotyledons*

Ethylene production was measured after the first 24 h. Chl data shown are means of eight samples ± SD after a 3-d incubation in the dark at 30°C. Identical letters indicate that means are similar at the 5% level.

Treatment	Ethylene Production	Chl Content	
		Air	10 μl/L ethylene
	$pl\ g^{-1}\ h^{-1}$	A_{665}/g fresh wt	
Initial, 0 d		24 ± 2 a	
Control	270 a	17 ± 2 c	9 ± 2 d
AVG, 1 μM	100 b	16 ± 2 c	11 ± 1 d
AVG, 10 μM	30 c	19 ± 3 bc	13 ± 4 d
AVG, 100 μM	30 c	21 ± 3 ab	10 ± 2 d

Figure 1 shows the difference in proteins isolated from initial time zero cotyledons (lane 1) and cotyledons exposed to air (lane 2) or 100 μl/L ethylene (lane 3) for 2 d. Ethylene and aging in air induced the formation of 30 to 33 kD and lower mol wt proteins compared to initial controls. Following procedures described in "Materials and Methods," two proteins with a red chromaphore (subsequently shown to be peroxidases) absorbing at 398 nm (see Fig. 4A later in "Results and Discussion") were isolated and purified by a combination of DEAE Bio-Gel A and Sephacryl S-200 and CM Bio-Gel A chromatography.

In Figure 1, lanes 4 through 7 show the effect of heating the 33-CPO and 60-CPO proteins on their mobility in SDS-PAGE. Lanes 4 and 5 show the electrophoretic patterns of 33-CPO and 60-CPO heated to 100°C for 3 min in the presence of SDS before electrophoresis. The unheated proteins were loaded in the absence of SDS in lanes 6 and 7. Heating the larger 60-kD protein resulted in the formation of a set of proteins with mol wt around 30 kD suggesting that 60-kD protein was either a dimer of two 30-kD subunits or that heating changed the configuration of the molecule so that it migrated more rapidly in the gel. Unheated 33-CPO, not treated with SDS, does not appear in the gel because it migrated to the negative electrode, which is consistent with its 8.9 pI (see below).

The possibility that some of the aging or ethylene-induced proteins represent nucleases was tested by running RNase and DNase activity gels (Fig. 2) with protein samples isolated from control and 100 μl/L ethylene-treated cotyledons. As shown in Figure 2, we were able to distinguish at least 7 bands of RNase and 6 bands of DNase activity, but failed to observe any difference between initials, air, and ethylene-treated cucumber seedlings.

The incorporation of $[^{35}S]Na_2SO_4$ into protein was used to test the idea that the increase in proteins in the 30- to 33-kD range represented protein synthesis. This experiment was based on the concept that the cell would convert the [35S]sulfate into labeled methionine and cysteine and that these amino acids would be incorporated into newly synthesized proteins. After a 3-d pretreatment with air or 100 μl/L ethylene to initiate synthesis of the red proteins, the cotyledons were excised and treated with $[^{35}S]Na_2SO_4$ for 24 h. The proteins were then extracted and run on the gels shown in Figure 3. In this composite, the Coomassie blue-stained protein gels are lined up next to the autoradiograms. The protein gels demonstrate the induction of the 33-kD protein by ethylene, and the autoradiograms show increased radioactivity in the corresponding region of the gel. In addition, the protein located at the 23-kD region of the gel also appears to be more radioactive as a result of the ethylene treatment. These experiments were repeated with [3H]leucine, and similar results were obtained (data not shown).

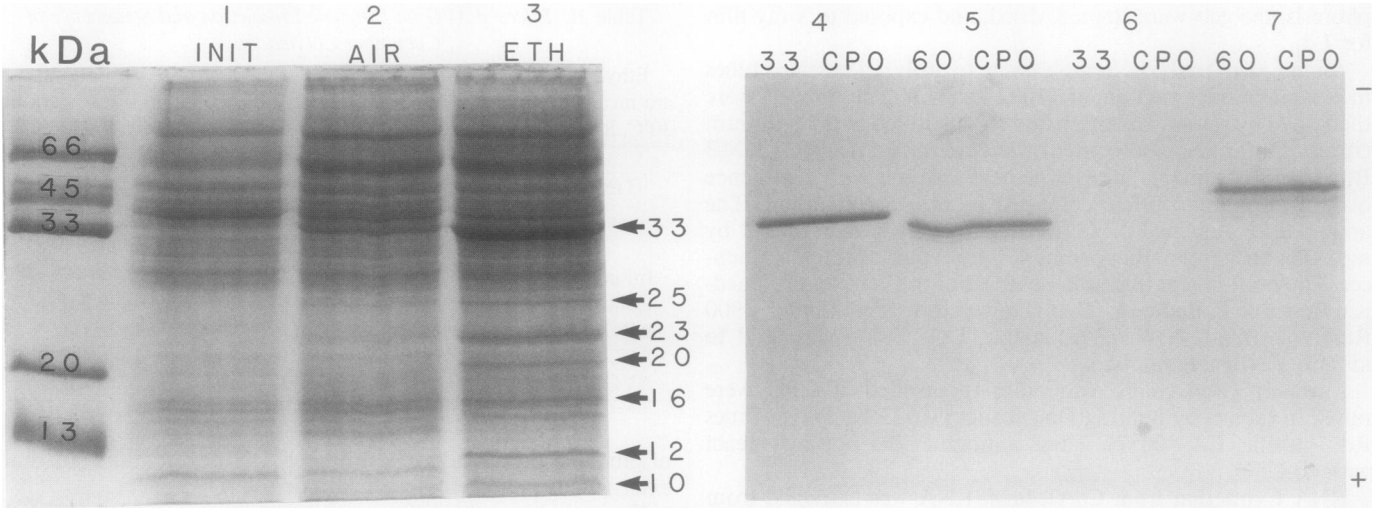

FIG. 1. Comparison of proteins isolated from 2-week-old cucumber cotyledons treated with 100 μl/L ethylene for 2 d. Samples loaded on lanes 6 and 7 were not heated or treated with 0.1% SDS before electrophoresis. All other samples were heated at 100°C for 3 min. Lanes: 1, 0 d initial; 2, 2 d air; 3, 2 d 100 μl/L ethylene; 4 and 6, 33-CPO; 5 and 7, 60-CPO. In this and subsequent figures, the position of mol wt markers is indicated on the right. Black arrows indicate proteins whose concentration was increased by ethylene treatment.

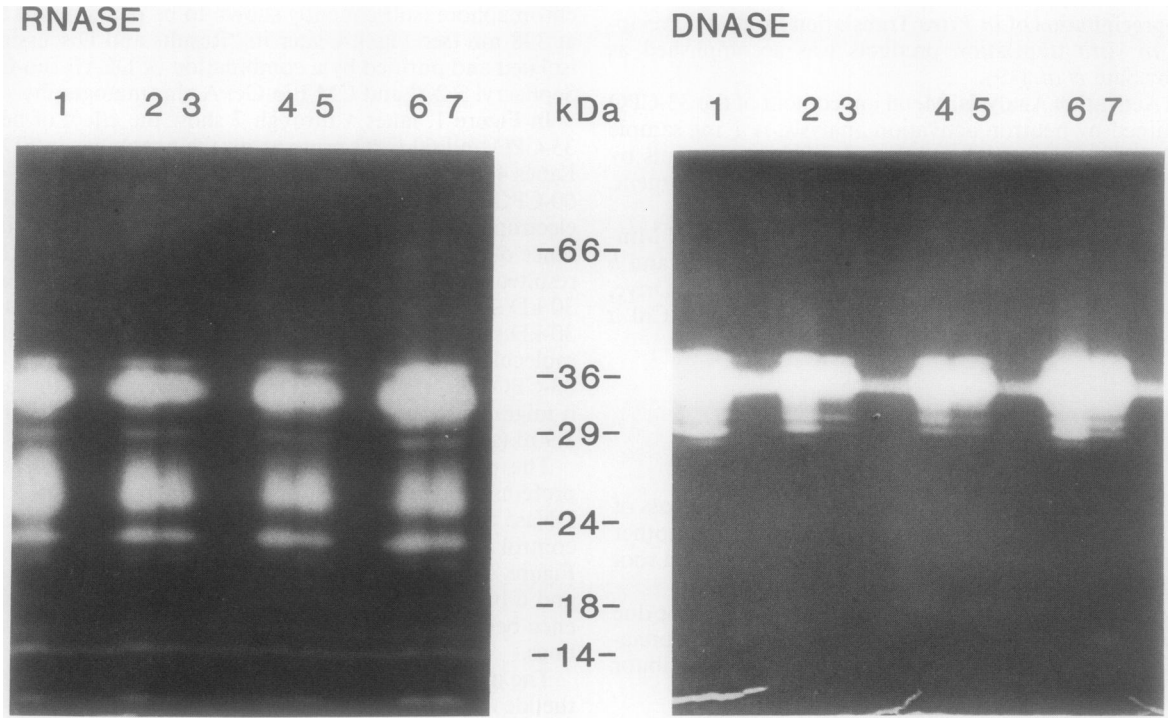

FIG. 2. RNase and DNase activity gels showing the lack of any apparent change in the induction or amount of nucleases during 100 ppm ethylene-induced senescence of cucumber cotyledons. For both gels, lane 1, initial; 2, 1 d air; 3, 1 d ethylene; 4, 2 d air; 5, 2 d ethylene; 6, 3 d air; 7, 3 d ethylene. See "Materials and Methods" for details.

The amino acid composition of the two proteins (subsequently shown to be peroxidases) is shown in Table III. Based on the amino acid composition, the minimum mol wt of the 33-kD protein was 26.5 kD and 60-kD protein was 48.4 kD. The difference between observed and calculated values was due in part to lack of data on the tryptophan content (destroyed in the acid hydrolysis procedure used to prepare samples) and the extent of protein glycosylation.

The absorption spectrum of purified 33 and 60-kD peroxidases is shown in Figure 4A and that of the chromaphores prepared by HPLC chromatography in Figure 4B. The absorption spectra appear to be similar and have three maxima, the largest at 398 and two smaller ones at 498 and 644 nm. Except for a sharpening of the 398 nm peak and a shift in the third peak from 644 to 626 nm, the spectrum of the chromaphores was the same as that of the intact proteins.

The presence of a large Soret band in the absorption spectra of the red proteins is similar to those of metalloporphyrins (20). Neutron activation analysis of the 33-kD protein indicated the presence of iron. Assuming the protein determination by the Bio-Rad protein reagent was correct, each mg of protein contained 0.722 ng of iron, which is equivalent to 0.42 mol of iron per mol protein. The mol ratio of other metals such as zinc,

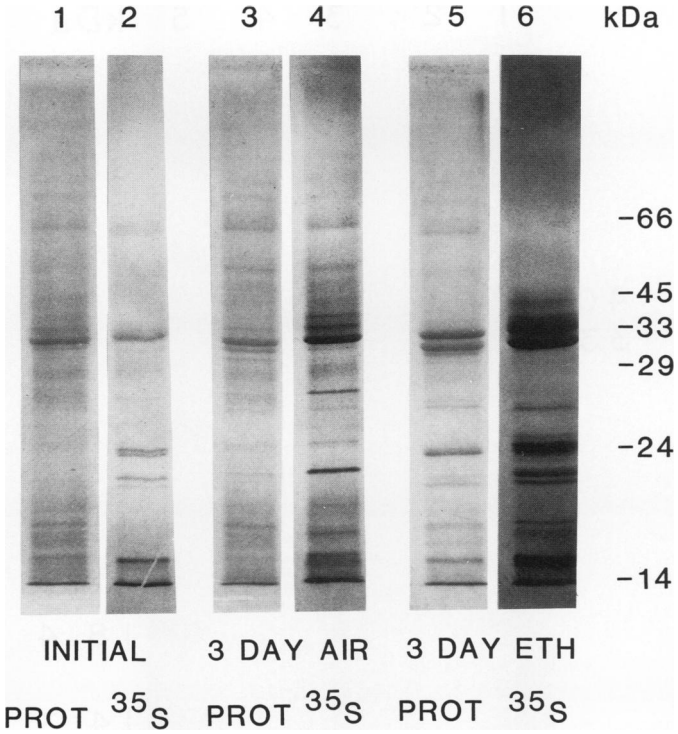

FIG. 3. Incorporation of [^{35}S]Na_2SO_4 into isolated cucumber cotyledon protein. Cotyledons were exposed to [^{35}S]Na_2SO_4 for 24 h and alcohol precipitable proteins isolated as indicated in "Materials and Methods." Cotyledons were harvested from 2-week-old untreated seedlings (lanes 1 and 2) or grown for an additional 3 d in either air (lanes 3 and 4) or 100 μl/L ethylene (lanes 5 and 6). In this composite, lanes 1, 3, and 5 are SDS-PAGE Coomassie blue-stained gels and lanes 2, 4, and 6 are the respective autoradiograms.

Table III. *Amino Acid Composition of 33 and 60 kD CPO*

Amino Acid[a]	Protein	
	33 kD CPO	60 kD CPO
	mol amino acid/mol protein	
Alanine	16	23
Arginine	14	28
Aspartic acid	31	43
Cysteine	5	11
Glutamic acid	21	25
Glycine	19	69
Histidine	3	15
Isoleucine	7	9
Leucine	16	36
Lysine	8	18
Methionine	1	4
Phenylalanine	12	14
Proline	8	1
Serine	18	35
Threonine	11	22
Tyrosine	3	6
Valine	9	16

[a] Tryptophan not determined. Values shown are the average of three analyses rounded out to the nearest whole number.

barium, and manganese was less than 0.03 mol metal ion per mol protein.

At this point of the investigation, the enzymic function of what were called red proteins was not known. An attempt was made to obtain information on the amino acid sequence of the 33-kD protein with the hope that it could be identified by

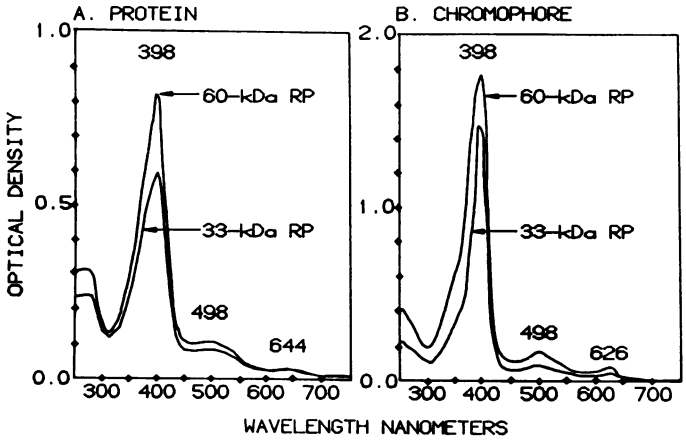

FIG. 4. Absorption spectra with absorption maxima of cucumber peroxidases. A, Absorption spectra of 33-CPO and 60-CPO at a concentration of 250 μg protein ml^{-1} water; B, absorption spectra of the chromaphores from 300 μg protein after separation from the polypeptide by HPLC. Chromaphores were dissolved in 1 ml of HPLC solvent consisting of 50% acetonitrile, 49.9% methanol, and 0.1% trifluoroacetic acid.

Table IV. *Comparison between the Amino Acid Sequence of a CNBr Fragment of 33-CPO and Other Plant Peroxidases (15)*

	170
Turnip peroxidase 7	... DMVALSGAHTIGQSRCVNF ...
	: : ::::::: : :: :
33-CPO CNBr fragment	... DLVSLSGAHTFGRSRNRFF ...
	::: ::: :::: ::
Horseradish peroxidase C	... DLVALSGGHTFGKNQCRFI ...
	170 180

: indicate amino acid homology. Numbers indicate location of the amino acid in the polypeptide chain.

comparing it with a data base of known proteins. The amino terminal end of the protein could not be analyzed because the terminal amino acid was modified and blocked. CNBr fragments of the 33-kD protein were prepared (21) and purified on an HPLC C_{18} column. Because the 33-kD protein has only one methionine, two fragments were formed. The partial amino acid sequence of one CNBr fragment is shown in Table IV. From a comparison with other proteins in the National Biomedical Research Foundation protein sequence database, it was found that the CNBr fragment showed some homology with the amino acid sequence of the internal part of horseradish C and turnip 7 peroxidase described earlier (14).

Figure 5 shows the results of separating cotyledondary and purified 33 and 60-kD proteins on isoelectric focusing gels. Location of proteins and peroxidase activity was indicated by Coomassie blue and guaiacol, respectively. The gels indicate the presence of a new protein and peroxidase with a pI of 8.6 in ethephon-treated cotyledons. The pIs of purified cucumber peroxidase were estimated as 8.6 for 33-CPO and 4.0 for 60-CPO.

Figure 6 shows the results of an *in vitro* translation and subsequent immunoprecipitation with rabbit anti-33-CPO of total RNA extracted from cucumber cotyledons treated with air or ethylene for 24 h. The autoradiogram of RNA translation products from the ethylene-treated cotyledons compared to air controls indicates the appearance of two new polypeptides at 40 and 33 kD. Immunoabsorption of the translation products from RNA that was extracted from ethylene-treated cotyledons with antibodies to 33-CPO resulted in the presence of a major poly-

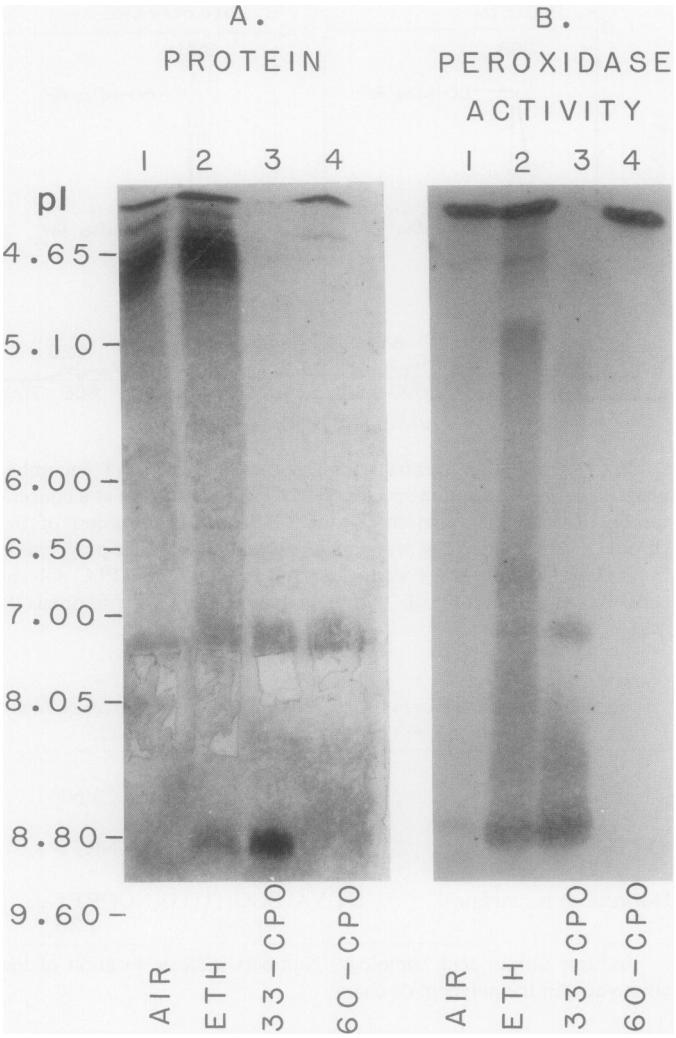

FIG. 5. Isoelectric focusing gels of 33-CPO, 60-CPO, and total alcohol precipitable proteins from cucumber cotyledons treated for 3 d with 20 mM ethephon. A, Coomassie protein stain; B, peroxidase activity stain. Lane 1, control cotyledons; 2, 3-d ethephon treated cotyledons; 3, 33-CPO; 4, 60-CPO. Position of pI standards indicated next to protein gel. Protein (μg) applied to lanes 1 through 4 in A was 40, 40, 4, 4 and to lanes 1 through 4 in B, 10, 10, 1, 1.

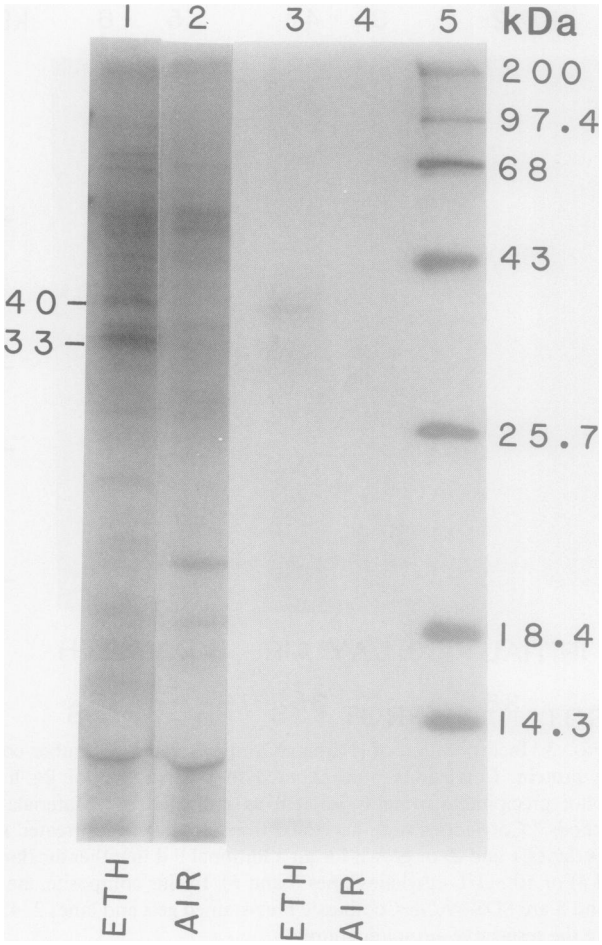

FIG. 6. Composite from autoradiogram of an *in vitro* translation and subsequent immunoprecipitation with rabbit anti-33-CPO of total RNA extracted from cucumber cotyledons treated with 100 μl/L ethylene or air control for 24 h. The autoradiogram of RNA translation products from the ethylene-treated cotyledons compared to air controls are shown in lanes 1 and 2, respectively. The location of two new polypeptides at 40 and 33 kD is indicated. The result of an immunoabsorption of translation products with antibodies to 33-CPO is shown in lanes 3 (ethylene) and 4 (air). Mol wt standards are shown in lane 5.

peptide band at 40 kD and a minor one at 33 kD. No polypeptides were precipitated with 33-CPO antibodies from the translation products of control RNA.

Table V presents data showing the degradation of Chl *a* and Chl *b* in a peroxidase degradation system (23). Horseradish peroxidase was used as a control. No loss in absorption was noted in the absence of enzyme or hydrogen peroxide (data not shown). We observed that all peroxidases tested in the complete system degraded both Chl *a* and *b*. In addition, 33-CPO and, to a lesser extent, horseradish peroxidase were capable of partially degrading Chl *a* in the absence of the flavinoid cofactor apigenin.

The change in the absorption spectra of Chl following peroxidative degradation in the system with apigenin is shown in Figure 7. The spectra obtained indicated that the degradation products still absorbed some light at the blue and red regions of the spectrum. The formation of pink catabolites described by Matile *et al.* (13) was not observed.

Table V. *In Vitro Degradation of Chl by Cucumber and Horseradish Peroxidase*

See "Materials and Methods" for experimental details.

Peroxidase	Apigenin	% Reduction in Absorbance after 10 min	
		Chl *a* (673 nm)	Chl *b* (650 nm)
Cucumber 33 kD	16 μM	61	57
	0	12	0
Cucumber 60 kD	16 μM	41	60
	0	3	1
Horseradish	16 μM	67	63
	0	8	0

CONCLUSIONS

The role of ethylene (17) and hydrolytic enzymes (8, 22) in senescence has been recently reviewed. Unlike the results presented by Lewington *et al.* (9), who reported increases in both RNase and DNase activity in senescing attached and detached cucumber cotyledons, we have observed that the ethylene-enhanced senescence of attached cucumber cotyledons was not accompanied by a change in nucleases as measured by enzyme activity gels. It is still possible that a nuclease could have escaped

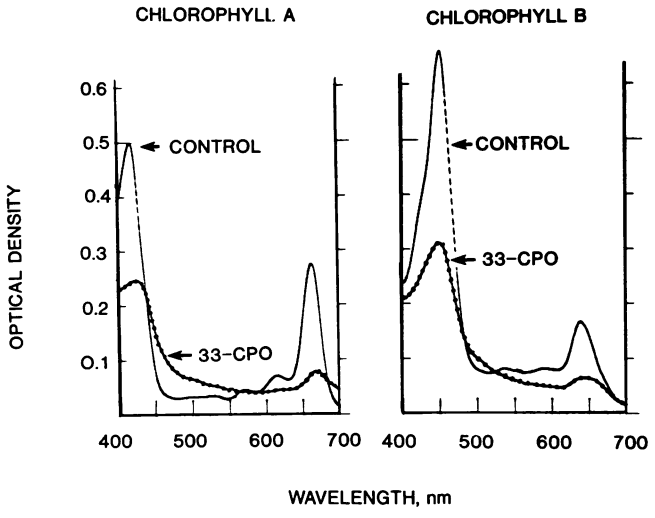

FIG. 7. Absorption spectra of Chl *a* and Chl *b* after degradation with 33-CPO. Reaction conditions were similar to those described for Table V except that the amount of Chl *b* used was increased three-fold.

detection because it was not active under the experimental conditions used here.

In the course of these experiments, we observed the induction of two proteins with identical red chromaphores during ethylene-enhanced senescence. The absorption maximum at the 398 nm region (Soret band) suggested the possibility that the chromaphore was a prophyrin, and the presence of iron suggested the porphyrin was a heme. However, the other two absorption maxima shown in Figure 7 did not match the spectral data of other porphyrins and metalloporphyrins summarized by Smith (20). Also, the lack of a clear 1 to 1 ratio of iron to protein raised the possibility that the iron was a contaminant or artifact of the isolation procedure.

Identification of the proteins became possible after the amino acid sequence of a CNBr fragment of the 33-kD protein was obtained. Comparison of the amino acid sequence of the CNBr fragment with polypeptides in the National Biomedical Research Foundation protein data bank suggested that the 33-kD protein was a peroxidase. Subsequent analysis for peroxidase activity as shown in Figure 5 confirmed the hypothesis that the red proteins were peroxidases. In addition, the data in Figure 5 suggest that the 60-kD peroxidase was a constitutive enzyme while the 33-kD peroxidase was induced.

The induction of 33-CPO during ethylene-induced senescence appears to meet some of the criteria for *de novo* protein synthesis. Earlier, Ford and Simon (6) showed the cycloheximide and actinomycin D inhibited the formation of peroxidase in excised cucumber cotyledons. As shown in Figure 3, [^{35}S]sulfate was incorporated into a protein with the same mol wt as 33-CPO. *In vitro* translation of RNA from ethylene-treated cotyledons indicated the presence of 40 and 33 kD translation products. Immunoprecipitation of these polypeptides with antibodies to 33-CPO is further evidence that the mRNA for this peroxidase was synthesized as a result of ethylene-induced senescence. The reason that both polypeptides react with antibodies to 33-CPO is not known. A possible explanation may be that the larger 40-kD polypeptide is an unprocessed precursor to the 33-kD polypeptide.

While these experiments suggest *de novo* synthesis of peroxidase during senescence, it is not possible to distinguish between a direct induction of the 33-CPO gene by ethylene or its activation by other processes or events set in motion as a result of ethylene-induced senescence.

The most obvious phenomenon occurring during leaf senescence is Chl loss, and a number of investigators have suggested that peroxidase plays a role in this process (12, 23). The observation that 33- and 60-CPO degrade Chl *in vitro* (Table V; Fig. 7) is consistent with this interpretation. However, Matile *et al.* (13) have shown that Chl breakdown may lead to the formation of pink degradation products with an absorption maximum of 562 nm, and such pigments were not formed in the *in vitro* Chl degradation system (Fig. 7). It is possible that peroxidases function as scavengers or general oxidases, and unspecifically catabolize Chl and other cellular components during senescence.

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