Plant Physiol. (1990) 92, 227–233
0032-0889/90/92/0227/07/$01.00/0

Received for publication April 19, 1989
and in revised form August 25, 1989

Effect of Ethephon on Protein Degradation and the Accumulation of 'Pathogenesis-Related' (PR) Proteins in Tomato Leaf Discs[1]

Pablo Vera* and Vicente Conejero

Departamento de Biotecnologia, Universidad Politécnica de Valencia, 46021-Valencia, Spain

ABSTRACT

The effect of ethephon (2-chloroetylphosphonic acid) on the degradation of proteins and on the induction of *Lycopersicon esculentum* pathogenesis-related (PR) proteins was studied in tomato leaf discs. The rate of ribulose,-1,5-bisphosphate carboxylase/oxygenase (Rubisco) degradation was maximal in discs after 48 hours of incubation with 1 millimolar ethephon, leading to complete disappearance of Rubisco after 96 hours. This effect was correlated with an increase in PR protein synthesis and the induction of the previously reported alkaline proteolytic enzyme PR-P69 (P Vera, V Conejero [1988] Plant Physiol 87: 58–63). *In vivo* pulse-chase experiments demonstrated that ethephon not only affected Rubisco content but that of many other ^{35}S-labeled proteins as well, indicating that ethylene activates a general and nonspecific mechanism of protein degradation. This effect was partially inhibited *in vivo* by the action of pCMB, a selective inhibitor of cysteine-proteinases such as P69. These data reinforce the hypothesis that P69 and perhaps other PR proteins are involved in the mechanism of accelerated protein degradation activated by ethylene.

A wide range of plants respond to pathogen attack or to different chemical treatments with the production of a characteristic set of proteins, the PR[2] proteins (25). This production seems to be mediated by ethylene (11, 25, 28). In tomato plants, the PR proteins fall into a group of basic proteins with mol wt ranging from 14.4 to 69 kD (11). The most abundant of these proteins P1(p14) is highly homologous to PR proteins from other plant species (17, 31).

Some PR proteins possess hydrolytic enzyme activities. Tomato P69 proteinase (26, 28), tobacco, and potato 1,3-β-glucanases and chitinases (13, 14, 16) are the best characterized so far. Function of PR proteins as proteinase and amylase inhibitors has also been reported (19).

Although the biological role of PR proteins in the response of plants to biotic or abiotic stress is not well understood, the extensive evolutionary conservation of PR protein production associated with the stress response suggests that the expression of PR proteins endows plants with a selective advantage.

Physiological and molecular analyses of PR proteins in plants have been performed primarily by extraction in acidic buffers (pH 2.8), which render most of other cellular proteins insoluble. To extend our analysis of stress-induced protein alterations, we have made extractions at a higher pH (7.4). At this pH, other cellular proteins are also solubilized, including Rubisco (EC 4.1.1.39). Rubisco is a major plant protein that is very stable and was, therefore, chosen as a model protein for these studies.

Our results show that PR protein accumulation after ethephon treatment is accompanied by a drastic degradation of other cellular proteins. Among these, Rubisco undergoes the most conspicuous degradation. In this paper, we show that the PR-P69 proteinase (26, 28) accounts for nearly 80% of the enhanced proteolytic activity induced by ethephon. These data suggest possible involvement of proteinase P69 and perhaps other PR proteins in ethylene-induced acceleration of protein degradation.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum* L. cv Rutgers) were cultivated in pots under natural light in a temperature controlled (25–30°C) greenhouse for 3 weeks after sowing.

Discs (2 cm in diameter) from expanded leaves sterilized in 1% sodium hypochlorite were cut in water, thoroughly washed with water, and blotted dry. The discs were floated on 20 mL sterile solutions in Petri dishes for the indicated times. The solutions consisted of different concentrations of ethephon, adjusted to pH 7.4 with 10 mM Tes buffer. At pH 7.4, chemical acid formation by ethephon is prevented, and ethylene is liberated at a much higher rate than at lower pH (5, 30, 32). At the indicated times, sets of five discs were homogenized in 50 mM Tris-HCl, 5 mM DTT (pH 7.2), with or without 1 mM PMSF and 1 mM pCMB in a 1:2 (w/v) ratio or in 84 mM citric acid-32 mM sodium phosphate, 20 mM 2-mercaptoethanol (pH 2.8), in a 1:1 (w/v) proportion. The homogenates were filtered and centrifuged for 20 min at 15,000*g*, and the supernatant fluids were used as the crude protein solution.

[1] Supported in part by grants from Comisión Asesora de Investigación Cientifica y Técnica (2509/84) and from Diputación de Valencia (Spain).

[2] Abbreviations: PR, pathogenesis-related; ethephon, 2-chloroethylphosphonic acid; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; FTC, fluorescein thiocarbamoyl derivative; pCMB, parachlormercuribenzoate; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone.

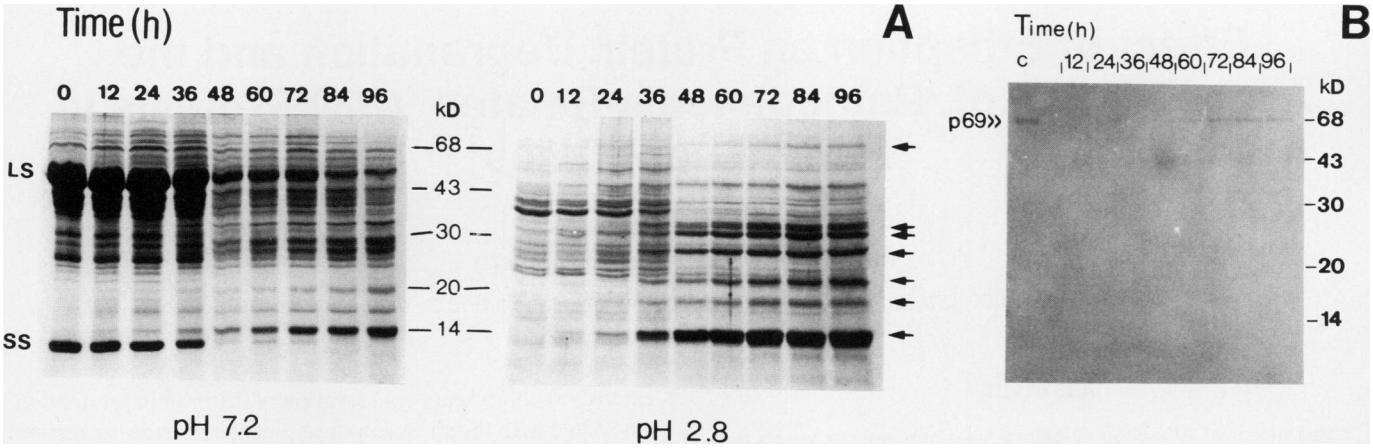

Figure 1. A, SDS-PAGE analysis of protein from tomato leaf discs treated with 1 mM ethephon for the indicated times (0–96 h). Left, Protein pattern obtained after extraction of leaf discs at pH 7.2. LS and SS indicate the position of Rubisco large and small subunit, respectively. Right, Protein pattern obtained after extraction of leaf discs at pH 2.8. The arrows denote the position of the PR proteins induced. Mol wt markers are indicated in the center of the figure. The SDS-gels were stained with Coomassie blue. B, Immunodetection of tomato proteinase P69 by Western blotting. Lane c corresponds to immunoblot of 0.4 μg of purified tomato proteinase P69. Lanes 12–96 correspond to immunoblots of equal amounts of crude extracts obtained from tomato leaf discs incubated with 1 mM ethephon for 12, 24, 36, 48, 60, 72, 84, and 96 h, respectively. Mol wt markers (kD) are indicated on the right.

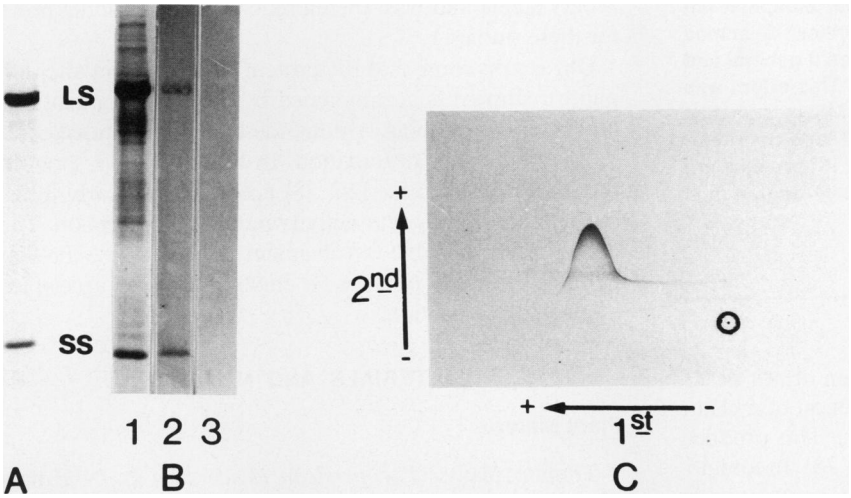

Figure 2. Specificity of Rubisco antibody. A, SDS-PAGE of a purified preparation of tomato Rubisco; B, characterization of Rubisco antibody by Western blot of a crude protein sample extracted at pH 7.2 and run on a SDS-PAGE (Coomassie blue staining): (1) transferred to nitrocellulose, and incubated either with Rubisco antisera (2) or with preimmune serum (3); C, cross-immunoelectrophoresis of a crude protein extract from tomato leaves run in agarose-embedded-Rubisco antiserum.

Isolation and Immunoassay of Rubisco

Isolation and purification of Rubisco were carried out as previously described (18, 26). Purified preparations of Rubisco were used for the preparation of specific antiserum. New Zealand rabbits were immunized by subcutaneous injection of 1 mL of a 1:1 emulsion of Rubisco (1 mg/mL) with complete Freund's adjuvant (GIFCO). After four booster injections with Rubisco in incomplete Freund's adjuvant at biweekly intervals, rabbits were sacrificed and serum was collected.

Specificity of the antisera against Rubisco was tested either by cross-immunoelectrophoresis or Western blotting of aliquots from tomato leaf extracts. Cross-immunoelectrophoresis (6) used 1% agarose gels buffered with barbital-buffer (Sigma). The second dimension involved 1% agarose (type II, Sigma) containing 1% (v/v) of Rubisco antiserum. Gels were run at 10 V/cm for 8 h in the same buffer.

Immunoblots were carried out as described (27). Proteins were separated by SDS-PAGE (14% acrylamide gels) and electrophoretically transferred to nitrocellulose membranes (0.45 μm pore size, Bio-Rad) as described by Towbin *et al.* (23). Nitrocellulose membranes were either stained with India-ink for transfer control (12) or blocked in blocking buffer (0.1 M Tris-HCl, 0.5 M NaCl, 0.01% Tween-20, 1% BSA [pH 7.4]) for 1 h. The blots were incubated with a 1:500 dilution of Rubisco antiserum in blocking buffer for 1 h and extensively washed for 1 h with three changes of 0.1 M Tris-HCl, 0.5 M NaCl, 0.01% Tween-20 (pH 7.4) (TBS). The blots were treated with anti-rabbit-IgG-sheep antibodies (Bio-Rad) labeled with horseradish peroxidase for an additional hour and washed as described above. Peroxidase activity was revealed with 4-chloro-1-naphthol (Sigma) dissolved in TBS containing 20% (v/v) methanol and 0.01% (v/v) H_2O_2 (0.5 mg substrate/mL).

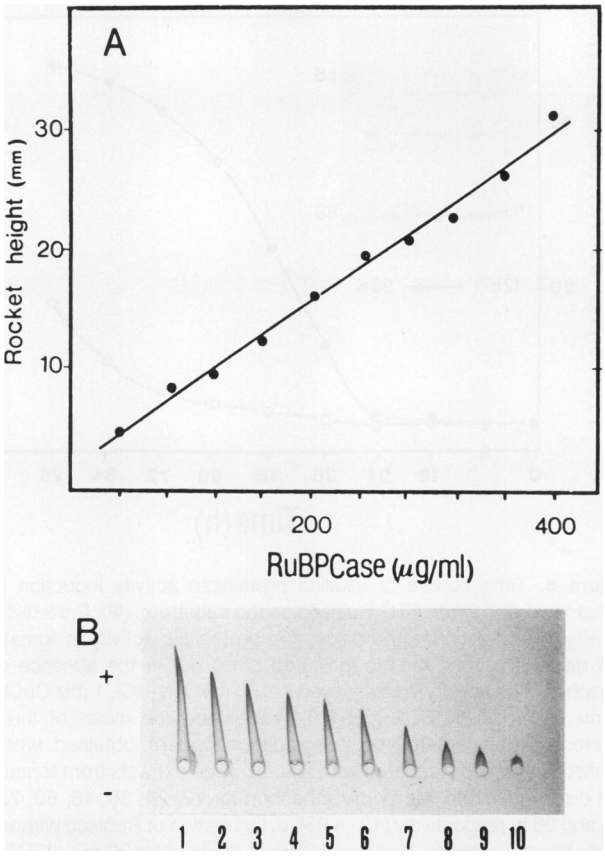

Figure 3. Quantification of Rubisco by rocket immunoelectrophoresis. A, Calibration curve obtained with standard concentrations of purified Rubisco; B, typical rockets obtained with the standard concentrations of Rubisco used to construct the calibration curve (numbers 1–10 represent 400–25 μg/mL of Rubisco, respectively).

Quantification of Rubisco

The concentration of Rubisco in crude extracts was determined by rocket immunoelectrophoresis (15) with Rubisco antiserum. Immunoelectrophoresis was performed in 1% agarose gels containing 1% (v/v) antiserum, at 10 V/cm for 12 h with barbital-buffer (pH 8.6). The Rubisco content in crude extracts was quantified by comparing it to a calibration curve of purified Rubisco.

PR-P69 Immunoblots

Proteinase P69 was detected by Western blotting with a specific polyclonal antibody obtained against purified proteinase P69 (28).

Immunoprecipitation of Proteinase P69 Activity

Immunoprecipitation of P69 proteinase activity was carried out as previously described (28). Briefly, 10 μL of either anti-P69 IgG or nonimmune IgG (0.5 mg/mL) were added to crude protein extracts (5 μL) to a final volume of 200 μL with immunoprecipitation buffer (100 mM Tris·HCl [pH 7.4], 0.2 M NaCl, 1% [v/v] Triton X-100). Samples were incubated for

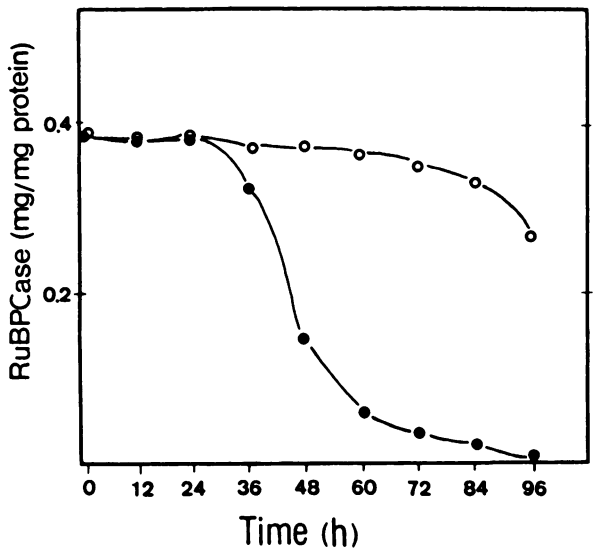

Figure 4. Effect of ethephon on Rubisco content in tomato leaf discs. Leaf discs were incubated either in the presence (●) or in the absence (○) of 1 mM ethephon. At the indicated times, the discs were homogenized, and the content of Rubisco was estimated by rocket immunoelectrophoresis. Point values are means of three independent experiments.

1 h at 37°C, then for 12 h at 4°C with shaking. Twenty μL of Protein A-Sepharose 4B (Pharmacia) (10 mg/mL) were added to each tube and incubated for 2 h at 25°C. Immunocomplexes were removed by centrifugation (10 min in a Beckman microfuge), and the supernatants were assayed for proteolytic activity as described below.

Electrophoretic Analysis

Proteins were analyzed by SDS-PAGE as described (7). Zymograms were performed as previously described (26, 28) in 14% SDS-PAGE prepared with 2 mg/mL fibrinogen (Sigma). Zymograms were incubated in the indicated buffers at room temperature for 12 h, and afterward SDS was removed by a 1-h incubation in 2% (v/v) Triton X-100 (26).

Protein Determination

Protein content in crude extracts was determined according to Bradford (4) with BSA as standard.

Enzyme Assay

Proteolytic activity was measured with FTC-Rubisco as a substrate, and the production of TCA-soluble fluorescent material was measured as described (26, 28). A typical reaction mixture contained 100 μL of the appropriate buffer, 30 μL of FTC-Rubisco (0.5 mg/mL) and 5 μL of crude extract. The solution was mixed thoroughly and incubated at 37°C for 20 min. Alternatively, the enzyme assay was carried out with Rubisco (unlabeled form), and the relative breakdown of Rubisco produced by 2 μL of crude extracts after incubation for 20 min in 0.1 M Tris-HCl, 1 mM DTT, 1 mM $CaCl_2$, 0.01%

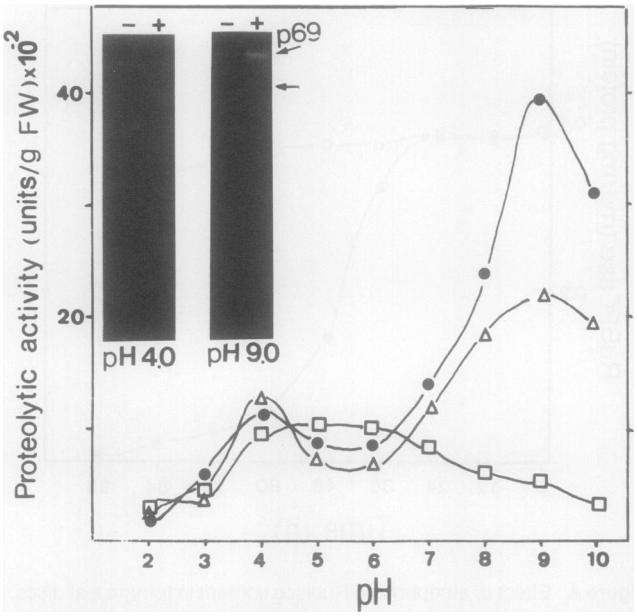

Figure 5. pH dependence for proteolytic activities induced by ethephon. Tomato leaf discs were incubated for 0 h (□), 40 h (△ . . . h (●) in the presence of 1 mM ethephon. Proteins were extracted and proteolytic activity was assayed with FTC-Rubisco as substrate as indicated in "Materials and Methods." The data were obtained in 0.1 M glycine-HCl buffer (pH 2–4), 0.1 M sodium acetate buffer (pH 5–6), 0.1 M Tris-HCl (pH 7–10), at 37°C. Inset, zymograms of crude extracts obtained from tomato leaf discs incubated either in the presence (+) or absence (−) of 1 mM ethephon for 96 h and assayed at pH 4.0 (left) or pH 9.0 (right). Equal amounts of crude extracts (20 μL) were assayed in each lane. Arrows denote the position of proteinase bands. Point values are means of three independent experiments.

(w/v) SDS, pH 9.0, was analyzed by SDS-PAGE after Coomassie blue staining as previously described (26).

In Vivo Labeling of Proteins in Tomato Leaf Discs

Tomato leaf discs (5 mm in diameter) were sterilized as described above, thoroughly washed with water for 20 min, and then labeled with L-(^{35}S)methionine (200 μCi/mL) (1000 Ci/mmol; Amersham) for 3 h. The discs were chased in 2 mM cold methionine in the presence or absence of 1 mM ethephon. Homogenization, either after 3 h pulse or upon chasing, was performed with 100 μL of cold 50 mM Tris-HCl, 5 mM DTT, 1 mM pCMB, 1 mM PMSF (pH 7.2), and supplemented with 10 μL of cold Rubisco (0.5 mg/mL). The extracts were centrifuged for 15 min in a Beckman microfuge at 4°C and supernatants subjected to SDS-PAGE. After electrophoresis, gels were stained with Coomassie blue, destained, and impregnated with Amplify (Amersham) prior to drying. Fluorography was performed on dry gels with Kodak XAR-5 at −80°C.

(^{35}S)Methionine incorporation into proteins was determined by counting the radioactivity of the cold TCA-insoluble proteins by liquid scintillation spectrometry.

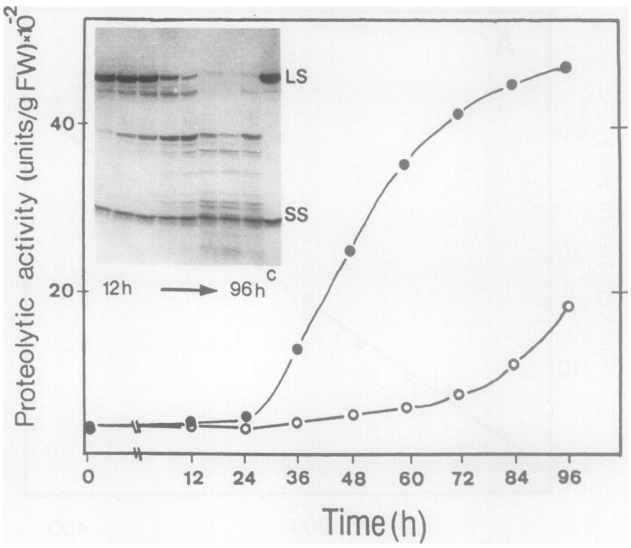

Figure 6. Time course of alkaline proteinase activity induction in tomato leaf discs with FTC-Rubisco as the substrate. (●), Proteolytic activity in ethephon-treated discs; (○), proteolytic activity in tomato leaf discs incubated for the indicated times but in the absence of ethephon. The activity was assayed in 100 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM DTT, 0.01% SDS (pH 9.0) (values are the mean of three different experiments). Inset, degradation pattern obtained when purified Rubisco is incubated with 2 μL of crude extracts from tomato leaf discs incubated with 1 mM ethephon for 12, 24, 36, 48, 60, 72, 84, and 96 h, respectively (12 → 96). c, Incubation of Rubisco without crude tomato extracts. Reactions were carried out for 20 min at 37°C in the buffer described above. LS and SS represent the position of the large and small subunits of Rubisco, respectively.

RESULTS

Protein Patterns after Ethephon Treatment

Changes in protein composition following ethephon treatment of tomato leaf discs were analyzed by SDS-PAGE (Fig. 1A). From the protein patterns obtained after extraction with a pH 7.2 buffer, two major polypeptides of mol wt 55 and 12.5 kD were identified as the large (LS) and small (SS) subunits of Rubisco, by comparison with purified tomato Rubisco (Fig. 2). During ethephon treatment, a decrease in Rubisco and an increase in PR proteins were observed. The increase in PR proteins was more evident when pH 2.8 was used for extractions (Fig. 1A). The decrease in Rubisco content was clearly observed after 36 to 96 h incubation of leaf discs in ethephon.

Ethephon concentrations ranging from 0.001 to 1 mM were tested in preliminary experiments (data not shown). A concentration of 1 mM was chosen for further experiments as the most effective in promoting the observed phenomena.

Measurement of Rubisco Content

A specific antibody raised against Rubisco holoenzyme, which recognized both subunits of the protein as shown by immunoblotting (Fig. 2), was used in these experiments. Rubisco content, determined by rocket immunoelectrophoresis (Fig. 3) in tomato leaf discs treated with ethephon, was

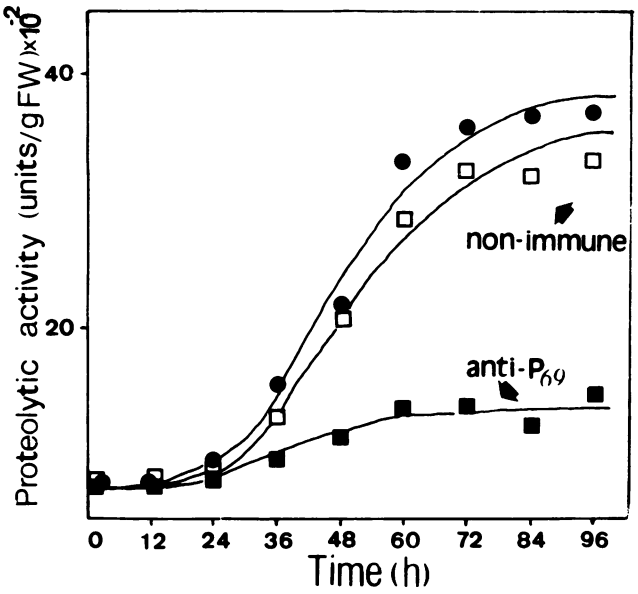

Figure 7. Inhibition of ethephon-induced proteolytic activity by antibodies against P69 proteinase. Tomato leaf discs were incubated with 1 mM ethephon for the indicated times, and the proteolytic activity was assayed with FTC-Rubisco as substrate and after crude extracts were treated with anti-P69 serum (■), nonimmune serum (□), or protein A-Sepharose alone (●) as described in "Materials and Methods."

compared with the content in buffer-treated control discs (Fig. 4). Rubisco content began to decrease significantly 36 to 48 h after ethephon treatment and was barely detectable at 96 h. The decrease in Rubisco was accompanied by the appearance of necrotic spots (at 60–72 h) in the discs, indicating cell death. By contrast, Rubisco content in control discs was stable up to 72 h, when a slight decrease was observed, probably caused by natural senescence of the discs.

Search for an Ethephon-Activated Proteolytic System

A search for proteolytic activities with capability to degrade Rubisco *in vitro* was performed with different pH buffers (pH 2–10). Rubisco was degraded in two pH regions (Fig. 5). At pH 8 to 10, 80% of total proteolytic activity was detected *in vitro* after 96 h of ethephon treatment. At pH 4.0, less than 10% of total proteolytic activity was detected.

Analysis by zymography (Fig. 5) revealed that the alkaline activity could be resolved into two proteinase bands, the most prominent representing the previously reported Ca^{2+}-stimulated alkaline proteinase PR-P69 (26, 28) and a lower mol wt band. PR-P69 was slightly induced in control discs after 96 h. No protease bands could be detected in zymograms at acidic pH (Fig. 5). A sharp increase in alkaline activity after 24 to 36 h of ethephon treatment was observed when FTC-Rubisco was used as substrate (Fig. 6). Degradation of the large subunit of purified Rubisco *in vitro* was demonstrated by SDS-PAGE (Fig. 6). The increase in alkaline activity was correlated with the accumulation of newly synthesized P69 protein, determined by immunoblotting of crude extracts with anti-P69 antiserum (Fig. 1B). Enhanced proteolysis was inhibited upon incubation of crude protein extracts with anti-P69 serum IgG and not by preimmune serum. These results suggest that the most active enzyme in the observed proteolysis is P69 proteinase (Fig. 7).

A useful way to determine which proteolytic system operates *in vivo* in intact cells is to use proteinase inhibitors. Cysteine proteinase inhibitor pCMB (0.05 mM), when added to the chase medium in the presence of ethephon, reduced the ethylene-induced protein degradation by 40 to 50% (Table I). Higher concentrations of pCMB were deleterious to the plant cells (data not shown). Proteinase inhibitors such as aprotinin, pepstatin, bestatin, leupeptin, 1,10-phenanthroline, and TLCK failed to prevent any significant protein degradation when added to the chase medium at a concentration of 0.05 mM (Table I).

Pulse-chase experiments, designed to detect loss of radioactivity incorporated into protein after ethephon treatment of leaf discs, revealed that Rubisco was degraded along with other proteins (Fig. 8).

DISCUSSION

The data presented in this paper describe two phenomena accompanying ethephon treatments of tomato leaf discs: (a) a progressive decrease on the contents of Rubisco and of other soluble proteins and (b) a progressive increase in PR proteins.

Ethylene released from ethephon solutions has been known to hasten several biochemical and physiological alterations, including proteolysis and other hydrolytic activities, the stimulation of oxidative enzymes, loss of Chl, and decline in photosynthetic rate. These alterations lead to early maturation and accelerated senescence (1, 2, 5, 10). *In vivo*, ethylene is involved in the response of plants to pathogens (3, 24). Ethylene mediation of viroid-induced developmental distortions and PR protein production has been reported (11). The viroid-induced PR protein P69, is an alkaline proteinase (26) and is inducible by ethephon (28).

Our observations suggest that the increased rate of degradation of Rubisco, as well as many other proteins, after ethephon treatments is caused by an ethephon-induced pro-

Table I. *Effect of Different Proteolytic Inhibitors on in vivo Degradation of (^{35}S)Methionine-Labeled Proteins*

Leaf discs were labeled with (^{35}S)methionine and chased for 50 h in the presence of 1 mM ethephon plus the indicated inhibitor (mean SEM; $n = 3$). Discs were homogenized and TCA-insoluble radioactivity was measured. Inhibition is expressed as % of initial (0 h of chase) cpm remaining as protein.

Inhibitor	Concentration	Initial cpm Remaining
	μM	%
None		14 ± 4.2
Bestatin	50	15 ± 7.0
Pepstatin	50	10 ± 2.1
Aprotonin	50	13 ± 9.3
Leupeptin	50	21 ± 0.6
TLCK	50	9 ± 1
pCMB	50	60 ± 3.6
1,10-Phenanthroline	50	9 ± 6.7

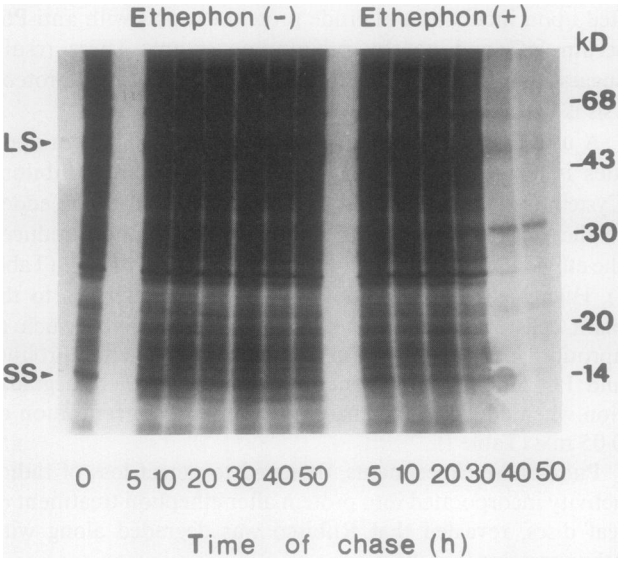

Figure 8. Effect of ethephon on the rate of *in vivo* degradation of proteins in tomato leaf discs. Tomato leaf discs were pulse-labeled with (^{35}S)methionine for 3 h and then chased for the indicated times in the presence (+) or in the absence (−) of 1 mM ethephon. Lane 0, time 0 h of chase; lanes 5–50, represent 5, 10, 20, 30, 40, and 50 h of chase. Arrows denotes the position of the labeled large and small subunits of Rubisco. Mol wt markers (kD) are indicated on the right. Fluorograms were exposed for 5 d.

teolysis, mostly resulting from the activity of PR-proteinase P69. This hypothesis is supported by the following facts: (a) The ethylene-induced alkaline proteolytic activity present in crude extracts cleaves Rubisco large subunit endoproteolytically. The proteolytic fragments generated are identical in mol wt to those reported (26) for *in vitro* degradation of purified Rubisco by P69 proteinase purified from viroid-infected leaves. (b) Like P69, this ethylene-induced proteolytic activity does not degrade Rubisco small subunit *in vitro* after a short incubation time (20 min). (c) Upon ethephon treatment, P69 accumulates progressively. (d) *In vivo*, pCMB, a selective inhibitor of cysteine proteinases (22), such as P69 (26), partially inhibits the degradation induced by ethephon. (e) *In vitro* degradation of Rubisco is inhibited by antibodies raised against P69 proteinase. (f) Our previous analysis of degradation of purified Rubisco with purified P69 (26) suggests that the level of proteinase P69 induced by ethephon is sufficient to completely degrade Rubisco.

In pulse-chase experiments, we have not been able to detect Rubisco proteolytic fragments equivalent to those produced by P69 in *in vitro* experiments (Fig. 6) (26). This suggests that, if those fragments are generated *in vivo*, they have a short life, and that a more complicated proteolytic system is in operation. In this regard, it is noteworthy that the proteolytic activity functioning at acidic pH (Fig. 5), and perhaps other proteolytic activities not detected here, may also be involved in the ethylene-induced response.

Since Rubisco is a very stable protein prior to senescence or stress (9, 18, 20), our findings indicate that ethylene induced stress and accelerated senescence. This was accompanied by PR protein synthesis and protein breakdown. These findings are consistent with our hypothesis that the ethylene-mediated response of tomato plants to viroid infection has a component of premature senescence (8, 27) that most probably leads to a cascade of events involved in cellular disassembly of the leaf as it occurs during natural senescence (21).

It is not yet possible to assign a precise role to the protease activities detected during aging of the leaf. However, a more detailed investigation of the acceleration of protein degradation observed after ethylene treatment would clarify the molecular mechanism(s) underlying this response. Furthermore, the search for the participation of other cellular factors (*e.g.* ubiquitin [29]) in the observed degradation of proteins may be significant and important.

ACKNOWLEDGMENT

We thank Dr. L. Perez Llorca for critical reading of the manuscript.

LITERATURE CITED

1. **Abeles FB** (1973) Ethylene in Plant Biology. Academic Press, New York
2. **Aharoni N, Lieberman M** (1979) Ethylene as a regulator of senescence in tobacco leaf discs. Plant Physiol **64:** 801–804
3. **Boller T** (1982) Ethylene-induced biochemical defenses against pathogens. *In* PF Wareing, ed, Plant Growth Substances. Academic Press, London, pp 303–312
4. **Bradford M** (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye-binding. Anal Biochem **72:** 248–254
5. **Choe HT, Whang M** (1986) Effects of ethephon on aging and photosynthetic activity in isolated chloroplasts. Plant Physiol **80:** 305–309
6. **Clarke MHJ, Freeman T** (1986) Quantitative immunoelectrophoresis of human serum proteins. Clin Sci **35:** 403–409
7. **Conejero V, Semancik JS** (1977) Analysis of the proteins in crude plant extracts by polyacrylamide gel electrophoresis. Phytopathology **67:** 1424–1426
8. **Conejero V, Picazo I, Segado P** (1979) Protein alterations in different hosts following viroid infection. Virology **97:** 454–456
9. **Friedrich JW, Huffaker RC** (1980) Photosynthesis, leaf resistances, and ribulose-1,5-bisphosphate carboxylase degradation in senescing barley leaves. Plant Physiol **65:** 1103–1107
10. **Gepstein S, Thimann KV** (1981) The role of ethylene in the senescence of oat leaves. Plant Physiol **68:** 349–354
11. **Granell A, Belles JM, Conejero V** (1987) Induction of pathogenesis-related proteins in tomato by citrus exocortis viroid, silver ions and ethephon. Physiol Mol Plant Pathol **31:** 83–89
12. **Hancock K, Tsang VCM** (1983) India ink staining of proteinase on nitrocellulose paper. Anal Biochem **133:** 157–162
13. **Kauffmann S, Legrand M, Geoffroy P, Fritig B** (1987) Biological function of "pathogenesis-related" proteins: four proteins of tobacco have 1,3-β-glucanase activity. EMBO J **6:** 3209–3212
14. **Kombrink E, Schroeder M, Hahlbrock K** (1988) Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc Natl Acad Sci USA **85:** 782–786
15. **Laurell CB** (1966) Quantitative estimation of proteins by electrophoresis in agarose gels containing antibodies. Anal Biochem **15:** 45–52
16. **Legrand M, Kauffmann S, Geoffroy P, Fritig B** (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754
17. **Lucas J, Camacho-Henriquez A, Lottspeich F, Henschen A, Sanger HL** (1985) Amino acid sequence of the "pathogenesis-related" leaf protein p14 from viroid-infected tomato reveals a new type of structurally unfamiliar protein. EMBO J **4:** 2745–2749

18. **Peterson LW, Huffaker RC** (1975) Loss of ribulose 1,5-diphosphate carboxylase and increase in proteolytic activity during senescence of detached primary barley leaves. Plant Physiol **55:** 1009–1015
19. **Richardson M, Valdes-Rodriguez S, Blanco-Labra A** (1987) A possible function for thaumatin and TMV-induced protein suggested by homology to a maize inhibitor. Nature **327:** 432–434
20. **Rosichand JL, Huffaker RC** (1984) Source of endoproteolytic activity associated with purified ribulose bisphosphate carboxylase. Plant Physiol **75:** 74–77
21. **Stoddart JL, Thomas H** (1982) Leaf senescence. *In* D Boulter, B Parthier, eds, Encyclopedia of Plant Physiology (New Series), Vol 14A. Springer-Verlag, Berlin, pp 592–636
22. **Storey RD, Wagner FW** (1986) Plant proteinases: a need for uniformity. Phytochemistry **25:** 2701–2709
23. **Towbin H, Staehlin T, Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA **76:** 4350–4354
24. **Van Loon LC** (1984) Regulation of pathogenesis and symptom expression in diseased plants by ethylene. *In* Y Fuchs, E Chalutz, eds, Ethylene: Biochemical, Physiological and Applied Aspects. Martinus Nijhoff/Dr W Junk Publishers, The Hague
25. **Van Loon LC** (1985) Pathogenesis-related proteins. Plant Mol Biol **4:** 111–116
26. **Vera P, Conejero V** (1988) Pathogenesis-related proteins of tomato. P69 as an alkaline endoproteinase. Plant Physiol **87:** 58–63
27. **Vera P, Hernández-Yago J, Conejero V** (1988) Immunocytochemical localization of the major "pathogenesis-related" (PR) protein of tomato plants. Plant Sci **55:** 223–230
28. **Vera P, Conejero V** (1989) The induction and accumulation of the pathogenesis-related P69 proteinase in tomato during citrus exocrotis viroid infection and after chemical treatments. Physiol Mol Plant Pathol **34:** 323–334
29. **Vierstra RD** (1987) Ubiquitin, a key component in the degradation of plant proteins. Physiol Plant **70:** 103–106
30. **Wagne HL, Leopold AC** (1969) Ethylene evolution from 2-chloroethyl phosphonic acid. Plant Physiol **44:** 156–158
31. **White RF, Rybicki EP, Von Wechmar MB, Dekker JL, Antoniw JF** (1987) Detection of PR-1-type proteins in *Amaranthaceae*, *Chenopodiaceae*, *Graminae* and *Solanceae* by immunoblotting. J Gen Virol **68:** 2043–2048
32. **Yang SF** (1969) Ethylene evolution from 2-chloroethylphosphonic acid. Plant Physiol **44:** 1203–1204