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Hydroxyl Radicals and a Thylakoid-Bound Endopeptidase are Involved in Light- and Oxygen-Induced Proteolysis in Oat Chloroplasts

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The hypothesis that light- and oxygen-induced proteolysis in chloroplasts is mediated by active oxygen species was examined. In order to determine whether or not H_2O_2 and/or $^{\cdot}OH$ radicals are involved in these degradative processes we compared the degradation of proteins in isolated oat chloroplasts exposed to white light at 80 W m^{-2} with that in chloroplasts incubated in darkness in the absence or presence of H_2O_2 or a $^{\cdot}OH$-generating system composed by ascorbic acid, $FeCl_3$ and H_2O_2 (Asc-Fe-H_2O_2). Light enhanced the rate of degradation of at least 18 polypeptides, while proteolysis was almost negligible in darkness in the abscence of additives. H_2O_2 had a very small effect. However, Asc-Fe-H_2O_2-treated chloroplasts in darkness showed a pattern of protein degradation almost identical to that observed in the light. A thylakoid-bound endopeptidase (EP), the activity of which increased under photooxidative environmental conditions and treatment with an $^{\cdot}OH$-generating system, was partially purified and characterized as a serine-type protease. Treatments with inhibitors of serine-type protease prevented both light- and Asc-Fe-H_2O_2-induced proteolysis. EP was more active against both soluble and membranous proteins that had been pretreated with Asc-Fe-H_2O_2 than against untreated proteins. It is proposed that a high dose of light irradiation promotes proteolysis by increasing the formation of $^{\cdot}OH$, which may modify proteins such that they become more susceptible to EP-catalyzed hydrolysis.

Key words: *Avena sativa* — Chloroplasts — Hydroxyl radicals — Oat — Protease — Proteolysis.

During senescence and under stressful conditions leaves progressively lose their photosynthetic capacity, as a consequence of the degradation of chloroplastic pigment and protein (Thomas and Stoddart 1980). Strong irradiation and/or extreme temperatures, when levels of O_2 are normal, promote a series of degradative changes within chloroplasts, among which the light-induced degradation and/or aggregation of the D1 protein and the subsequent inhibition of the PSII are among the most widely studied reactions at the present (Jegerschöld et al. 1990, Kyle 1987, Roberts et al. 1991). Under strong irradiation, in particular under CO_2-limiting conditions, O_2 becomes an alternative acceptor of photosynthetically transported electrons and is transformed into H_2O_2, O_2^-, $^{\cdot}OH$ and $^1O_2$ (Asada and Takahashi 1987, Robinson 1988), all of which seem to play important roles in the hydrolysis of the D1 protein of PSII (Bradley et al. 1991, Kyle et al. 1984, Krause and Cornic 1987). Furthermore, there is direct and indirect evidence to indicate the involvement of active oxygen species, generated under photooxidative conditions, in the increased rate of breakdown of other chloroplastic proteins (Casano et al. 1990, Casano and Trippi 1992), such as ribulose bisphosphate carboxylase/oxygenase (RuBisCO) (Weckenman and Martin 1984). Even though there seems to be a consensus with respect to the involvement of active oxygen, the current literature about the relative importance of each active compound in light- and oxygen-induced proteolysis is far from definitive. Indeed, Bradley et al. (1991) recently reported that H_2O_2 can mediate photoinhibition of PSII, while Weckemann and Martin (1984) found that the rate of degradation of RuBisCO increases when the protein has previously been modified by exposure to a system that

Abbreviations: Asc-Fe-H_2O_2, ascorbic acid-$FeCl_3$-H_2O_2 system; EP, thylakoid-bound endopeptidase; Na-P_i, sodium phosphate, $^{\cdot}OH$, hydroxyl radical; PMSF, phenylmethylsulfonyl fluoride; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; $^1O_2$, singlet oxygen; O_2^-, superoxide ion; TLCK, tosyl-lysine chloromethyl ketone.

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generates $^1O_2$. In addition, previous results from our laboratory indicate that $^{\cdot}OH$ may be one of the most important active species of oxygen involved in the rapid proteolysis observed in oat chloroplasts under photoxidative conditions (Casano and Trippi 1992).

Oat chloroplasts contain at least four proteolytic activities (Casano et al. 1990), but the level of only one, probably that of a thylakoid-associated endopeptidase (EP), increases when plastids are subjected to strong irradiation (Casano et al. 1990) or to treatment with active oxygen in darkness (Casano and Trippi 1992). Moreover, in isolated oat chloroplasts, $^{\cdot}OH$ scavengers such as mannitol (Casano and Trippi 1992) and benzoate (Casano, unpublished data) prevented both light-induced proteolysis and the correlated increase of EP activity. However, there is as yet no conclusive evidence with respect to the involvement of this protease in the process under study. Recently, Virgin et al. (1991), working with protease inhibitors, showed that a serine-type protease could catalyze the light-induced degradation of D1 protein.

The nature of chemical changes that result from the action of active oxygen species on chloroplast proteins and their consequences on the light- and oxygen-promoted proteolysis remain unclear. Employing both crude extracts from erythrocytes and isolated animal proteins, Davies and his group were able to demonstrate that $^{\cdot}OH$ and O_2^- can cleave polypeptides directly, while $^{\cdot}OH$ alone can modify proteins to render them more susceptible to proteolytic attack (Davies 1987, Davies et al. 1987). O_2^- by itself does not have any detectable effect on the breakdown of proteins (Davies 1987). Differences in susceptibility to active-oxygen-induced degradation have been obseved in proteins from both animal cells (Davies 1987) and chloroplasts (Casano and Trippi 1992).

The aim of the present work was to gain some insight into the role of active oxygen in light-induced proteolysis in chloroplasts by characterizing the active oxygen compounds involved in the process under study and the nature of EP and its regulation by active oxygen. The results presented here strongly suggest that the $^{\cdot}OH$ radical is, at least, one of the most important oxygen species that mediate light-induced proteolysis. The thylakoid-associated EP, which was partially purified and characterized, seems to be the main enzyme that catalyzes the hydrolysis of proteins under photooxidative conditions, in paticular those damaged by oxygen radicals.

Materials and Methods

Isolation of chloroplasts and of soluble and membrane fractions derived from them—Intact chloroplasts were obtained from the first leaves of 7- to 9-day old oat plants as previously described (Casano et al. 1990), and they were resuspended with 50 mM sodium phosphate (Na-P_i) buffer (pH 8.0) that contained 0.4 M sucrose. Integrity of chloroplasts was estimated by the ferricyanide photoreduction assay, and suspensions with fewer than 75% intact chloroplasts were discarded. Stromal proteins were separated from thylakoidal proteins by exposure of intact chloroplasts to 50 mM Na-P_i buffer, pH 8.0, which resulted in hypotonic shock. After centrifugation of this preparation for 10 min at $14,500 \times g$, the pellet was washed twice with the same buffer supplemented with 150 mM NaCl. The first two supernatants were pooled and used as the soluble protein fraction. The pellet was resuspended in 50 mM Na-P_i buffer, pH 8.0, and used as thylakoid-associated proteins. Both intact chloroplasts and membranes were brought to 0.4 mg Chl ml^{-1} in the respective resuspension media. All procedures were carried out at 4°C.

Analysis of the effect of light and active oxygen on proteolysis in chloroplasts—Intact chloroplasts (2.5 mg protein ml^{-1}) were incubated for 0 and 2 h at 24°C in darkness and in the presence of (a) 0 mM, 0.5 mM or 5.0 mM H_2O_2 (final concentrations), and (b) a mixture that generated $^{\cdot}OH$ radicals composed by 0.5 mM ,0.05 mM and 0.5 mM or 5.0 mM, 0.5 mM and 5.0 mM (final conc.) of ascorbic acid, $FeCl_3$ and H_2O_2 (Asc-Fe-H_2O_2), respectively, dissolved in the resuspension medium. The final volume of the reaction mixture was 200 μl. The amount of $^{\cdot}OH$ produced by the Asc-Fe-H_2O_2 system was estimated by monitoring the cleavage of methionine in the presence of pyridoxal phosphate, as described by Youngman and Eltsner (1981), and about 12 ng h^{-1} of ethylene (the product of the $^{\cdot}OH$-mediated cleavage of methionine) were produced by a mixture that contained 5.0 mM ascorbic acid, 0.5 mM $FeCl_3$ and 5.0 mM H_2O_2 at 25°C. Simultaneously, samples of chloroplasts were incubated in the light (80 W m^{-2}) for 0 and 2 h at 24°C. At the indicated times, approximately 20 μg of chloroplast proteins were subjected to electrophoresis in 5%/12% polyacrylamide gels under denaturing conditions, as described by Laemmli (1970). Polypeptides were stained with Coomassie Brilliant Blue.

Partial purification and characterization of the thylakoid EP. Purification of the protease—The entire procedure consisted of three steps, as follow: (i) isolation of thylakoids, (ii) acetone precipitation and (iii) affinity chromatography on hemoglobin-agarose gel. One hundred g of primary leaves of 7-days-old oat plants were homogenized for 10 s at maximum speed in a Sorvall Omni-mixer (Dupont, CT, U.S.A.) with 250 ml of a medium that contained 50 mM Tris-HCl (pH 7.8), 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 0.2% bovine serum albumin. After filtration through four layers of Miracloth, debris was removed by centrifugation at $1,000 \times g$ for 1 min. The supernatant was again pelleted at $6,000 \times g$ (7 min) and pelleted thylakoids were washed twice with 50 mM Na-P_i buffer, pH 8.0, that contained 150 mM NaCl. Washed thylakoids were resuspended in 50 mM Na-P_i buffer, pH 8.0. The separation

of the EP from thylakoids was initially performed by treating membranes with three volumes of cold acetone at −20°C. After constant stirring for 30 min, precipitated proteins were pelleted by centrifugation at 5,000 × *g* for 10 min, washed twice with cold acetone and dried under nitrogen gas. The acetone powder, resuspended in 50 mM Na-P_i buffer, pH 8.0, that contained 0.1% Triton X-100 (buffer A), was loaded onto a column (1.6 cm i.d. × 5 cm) of hemoglobin-agarose (obtained from SIGMA Co., St. Louis, MO, U.S.A.) that had been equilibrated with the same buffer. Unbound proteins were separated from the matrix by washing the column with buffer A and then EP was eluted by changing the buffer to 2 M NaCl dissolved in buffer A. Peak fractions (1 ml each) were pooled and the preparation was designated isolated EP.

Dependence of pH of EP activity—Ten-μl aliquots of either a suspension of thylakoids (5 μg of protein) or isolated EP (0.2 μg of protein) were incubated with 50 μg hemoglobin dissolved in 100 μl of 100 mM citrate-P_i buffer over a range of pH values from 4.0 to 6.5 for 30 min at 39°C. EP activity was estimated by measuring the decrease in the amount of undigested proteins, as described elsewere (Casano et al. 1990). In routine assays, EP was assayed at pH 4.8.

Effects of protease inhibitors—Isolated EP and thylakoids were preincubated with different protease inhibitors at the indicated concentrations for 1 h at 4°C and then residual activity was assayed by the standard procedure.

In other experiments, aliquots of thylakoids (20 μg of protein), resuspended in 100 mM citrate-P_i buffer, pH 4.8, were preincubated with 1 mM PMSF and 0.1 mM TLCK for 1 h at 4°C, and then they were subjected to light (80 W m^{-2}), dark and Asc-Fe-H_2O_2 treatments for 2 h at 24°C. Results were analyzed by SDS-PAGE as described above.

Influence of the concentration of substrate on EP activity—EP activity of either thylakoids or the isolated enzyme was assayed in the presence of hemoglobin at different concentrations, from 0 to 1.0 mg ml^{-1}, at pH 4.8, as described above.

In other experiments, thylakoids were pretreated with either 5.0 mM H_2O_2 or 5.0 mM ascorbic acid, 0.5 mM $FeCl_3$ and 5.0 mM H_2O_2 for 1 h at room temperature and then the influence of the concentration of the substrate on EP activity was assayed as described above.

EP activity against oxidatively damaged proteins of oat chloroplasts—Aliquots of 1.5 mg of either soluble or membrane-associated proteins (thylakoids, see above) from oat chloroplasts were incubated in the presence of the Asc-Fe-H_2O_2 system (in 100 mM Na-P_i buffer, pH 8.0) with the following final concentrations of ingredients: 0 mM, 0 mM and 0 mM; 2.0 mM, 0.2 mM and 2.0 mM or 10.0 mM, 1.0 mM and 10.0 mM, ascorbic acid, $FeCl_3$ and H_2O_2, respectively, at room temperature in darkness. The final volume of each reaction mixture was 0.7 ml. After 1 h, 150-μl samples of each mixture were incubated with or without isolated EP (100 μl of a preparation of 0.1 mg protein ml^{-1}) and 6 μl of 0.5 M citric acid (in order to shift the pH to 4.8) for 2 h at 39°C. Reactions were stopped by addition of trichloroacetic acid to 4.5% (final concentration). After 30 min in an ice bath, undigested or large polypeptides were removed by centrifugation for 10 min at 14,500 × *g*. Products of proteolysis (amino acids or small peptides that were soluble in trichloroacetic acid) were quantified by measuring the absorbance at 280 nm (A280 nm). In other experiments, reactions were stopped with the sample buffer for SDS-PAGE, and samples of 20 μg of protein were subjected to electrophoresis in an acrylamide gel under denaturing conditions, as described above.

Quantification of chlorophyll and protein—Chlorophyll was quantified by the method of Wathley and Arnon (1963) and proteins by the technique of Sedmark and Grossberg (1977) with bovine serum albumin as standard.

All the experiments were repeated at least three times and representative data are presented.

Results and Discussion

The hydroxyl radical is involved in light-induced proteolysis in isolated oat chloroplasts—Even though both H_2O_2 and ·OH have been shown to be able to enhance the hydrolysis of isolated proteins from different sources (Bradley et al. 1991, Casano and Trippi 1992, Davies et al. 1987), the actual involvement of these active species of oxygen in light-induced proteolysis in chloroplasts is still obscure. In the present study we compared the effects of H_2O_2 and an ·OH-generating system (Asc-Fe-H_2O_2) on proteolysis in chloroplasts in the dark with that in the light. As expected, a 2-h incubation under illumination at 80 W m^{-2} promoted the significant degradation of many chloroplast polypeptides while proteolysis was barely detected in darkness (Fig. 1). However, treatment with Asc-Fe-H_2O_2, at the maximum level, of chloroplasts in darkness strongly enhanced proteolysis (Fig. 1). Even though the Asc-Fe-H_2O_2 system contains H_2O_2 its effect should be attributable to ·OH radicals, since when chloroplasts were exposed to H_2O_2 at the same concentrations as those present in Asc-Fe-H_2O_2 systems, only a slight increase in the rate of degradation of proteins was observed (Fig. 1). Moreover, the mixture of 5.0 mM ascorbic acid, 0.5 mM $FeCl_3$ and 5.0 mM H_2O_2 actually produced ·OH radicals, as demonstrated by the ·OH-induced cleavage of methionine (see Materials and Methods) while neither ascorbic acid plus $FeCl_3$ nor H_2O_2 alone had any detectable effect on the stability of methionine (data not shown). In a previous study (Casano and Trippi 1992) we found that an ascorbic acid-$FeCl_3$ system (without H_2O_2) increased the degradation of seven polypeptides in isolated oat chloroplasts. However, this effect attributed to the ascorbic acid-$FeCl_3$-

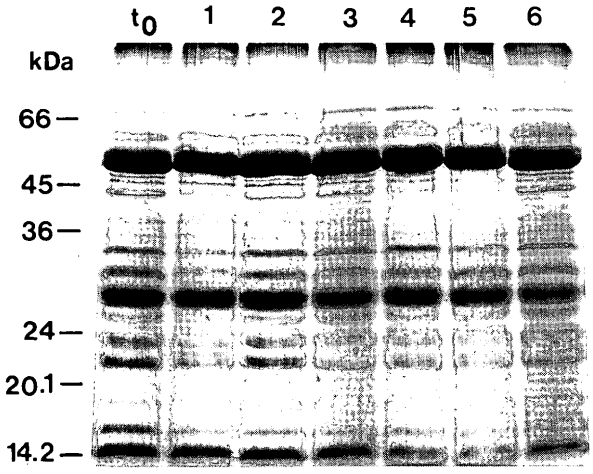

Fig. 1 Effects of light, hydrogen peroxide and ·OH radicals on proteolysis in isolated oat chloroplasts. Isolated plastids (2.5 mg proteins ml^{-1}) were incubated in 50 mM Na-P_i buffer, pH 8.0, and 0.4 M sucrose for 0 (t_0) and 2 h at 24°C under the following conditions: white light at 80 W m^{-2} plus water (1), darkness plus 0.5 mM H_2O_2 (2), darkness plus 5.0 mM H_2O_2 (3), darkness plus 0.5 mM ascorbic acid–0.05 mM $FeCl_3$–0.5 mM H_2O_2 (4), darkness plus 5.0 mM ascorbic acid–0.5 mM $FeCl_3$–5.0 mM H_2O_2 (5) and darkness plus water (6). Volumes of added water, H_2O_2 and Asc-Fe-H_2O_2 system were less than 2% of the final volume of the incubation mixture. Reactions were stopped with SDS sample buffer, and 20 μg of proteins were subjected to electrophoresis under denaturing conditions (see Materials and Methods). Polypeptide bands were stained with Coomassie Brilliant Blue.

induced formation of ·OH radicals, was smaller than that of the present Asc-Fe-H_2O_2 system (Fig. 1). It is probable that the ability of the ascorbic acid-$FeCl_3$ system to form ·OH radicals was limited by the availability of endogenous H_2O_2, which was presumably very low in chloroplasts in darkness, since the addition of catalase abolished the effects of both ascorbic acid-$FeCl_3$ and Asc-Fe-H_2O_2 systems (data not shown). Our semiquantitative analysis of patterns of polypeptide degradation revealed significant differences in susceptibility to active oxygen species among chloroplastic proteins. It is of interest to note here that the hydrolysis of a 32-kDa polypeptide was preferentially promoted by H_2O_2, as reported by Bradley et al. (1991) (Table 1). However, a clear similarity between the effects of light and ·OH radicals was observed, from both a quantitative and a qualitative point of view (Table 1, Fig. 1), indicating that the ·OH radical plays a major role in the photooxidative promotion of proteolysis in chloroplasts.

Partial purification and characterization of a thylakoid-associated protease, its involvement in light- and active-oxygen-induced proteolysis—In previous studies (Casano et al. 1990, Casano and Trippi 1992), we observed that a thylakoid-bound "acid" endopeptidase activity in-

Table 1 Semiquantitative comparison of the effects of light and darkness plus hydrogen peroxide or a hydroxil radical-generating system on protein degradation in isolated oat chloroplasts

kDa	Light	H_2O_2 (Darkness)	·OH (Darkness)
68	×	—	×
65	×	—	×
62	××	—	××
60	××	—	××
55	×	—	×
49	××	—	××
45	××	×	××
43	×	—	××
39	×	—	××
37	—	—	××
35	—	—	×
33	×	×	—
32	××	××	×
29	×	—	×
27	××	×	××
24	××	×	×
23	—	×	×
20	×	×	×
17	×	×	×
16	×	×	×
15	×	—	×

Isolated chloroplasts were exposed to light or to 5.0 mM H_2O_2 or to 5.0 mM ascorbic acid–0.5 mM $FeCl_3$–5.0 mM H_2O_2 in darkness for 2 h. Residual polypeptides were separated by SDS-PAGE and stained as described in Materials and Methods. The rate of degradation of each polypeptide was estimated by comparing the densitograms before and after 2 h of treatment. (—) indicates a degradation rate of less than 10%; (×), a degradation rate between 11% and 50% and (××), a degradation rate above 51%.

creased under both photooxidative conditions and upon treatments with active oxygen species. With the aim of characterizing the EP activity, we partially purified the enzyme from isolated thylakoids employing acetone precipitation and delipidization, which was followed by chromatography on an affinity column of hemoglobin-agarose. As shown in Figure 2, no EP activity was found in the eluate when the column was washed with equilibrating buffer, but the enzyme was eluted in the presence of 2 M NaCl. This procedure yielded a preparation of EP that was not homogeneous (as analyzed by SDS-PAGE, data not shown). However, the extent of purification (Table 2) was considered sufficient for studies of some characteristics of the enzyme.

Isolated EP exhibited its maximum activity at pH 4.5 (Fig. 3). This behaviour of the enzyme was very similar to that observed in isolated chloroplasts and thylakoids, in

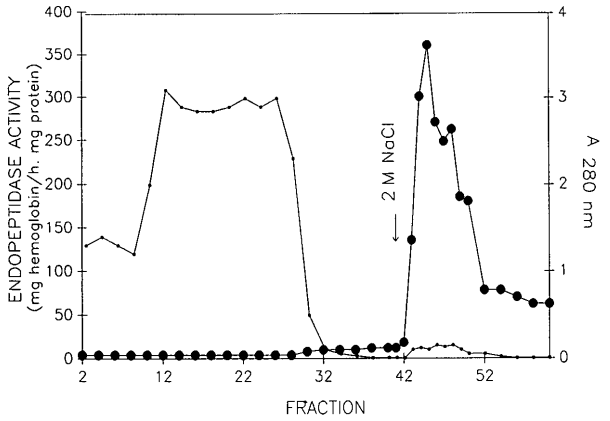

Fig. 2 Isolation of an "acid" endopeptidase from oat thylakoids by affinity chomatography. After acetone precipitation and resuspension in 50 mM Na-P_i buffer, pH 8.0, and 0.1% Triton X-100, thylakoid proteins were loaded onto a column of hemoglobin-agarose that had been equilibrated with the same buffer. The column was washed with equilibration buffer and then the protease was eluted with 2 M NaCl. EP activity, (-●-); A280, (-·-).

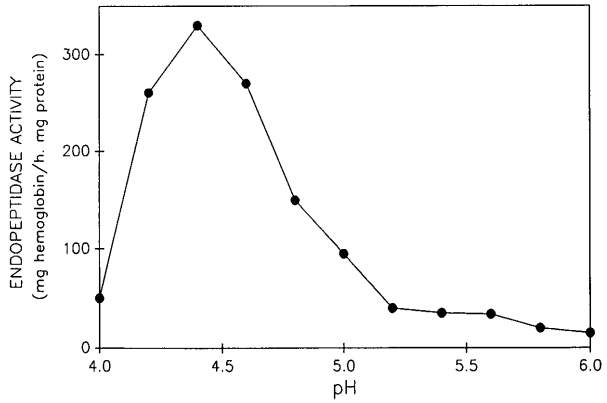

Fig. 3 Influence of pH on the activity of the "acid" endopeptidase isolated from oat thylakoids. Aliquots of isolated EP (2 μg of protein) were incubated with 50 μg of denatured hemoglobin that had been dissolved in 100 μl of 100 mM citrate-P_i buffer over a range of pH values from 4.0 to 6.5, for 30 min at 39°C. Reactions were stopped by addition of 3 ml of 0.2% Coomassie Brilliant Blue G-250 dissolved in 30% ortho-phosphoric acid. EP activity was estimated by monitoring the decrease in levels of undigested proteins, as measured at 595 nm.

which EP activity was highest at pH 4.8 (Casano et al. 1990). Changes in the environment of the enzyme might account for the minor difference in dependence on pH between the isolated and membrane-associated EP. In agreement with our results, Tang and Huffaker (1984) reported that thylakoids of barley contain a peptidase that is active at low pH.

The effects of protease inhibitors on EP activity were assayed with hemoglobin as substrate and both the isolated enzyme (Table 3) and thylakoids (data not shown). In both cases, neither inhibitors of cysteine-type proteases (*N*-ethylmaleimide and iodoacetamide) nor an inhibitor of aspartyl-type proteases (pepstatin) or inhibitors of metal-type proteases (EDTA and *o*-phenanthroline) had any significant effect (Table 3). However, phenylmethylsulfonyl fluoride (PMSF) and tosyl-lysine chloromethyl ketone (TLCK) inhibited the EP activity of both the isolated enzyme and the thylakoids, indicating that the EP might be a serine-type protease. Kawasaki and Takeuchi (1989) found that a thylakoid-associated, serine-protease-like character (revealed as a diisopropylfluorophosphate-binding protein) increased markedly in senescent spinach leaves. More recently, Virgin et al. (1991), working with protease inhibitors, demonstrated that the hydrolysis of the D1 protein of spinach thylakoids is catalyzed by a serine-type protease, which is probably associated with PSII. On the other hand, the protease examined in the present study was distinct from the dithiothreitol-sensitive protease purified by Kuwabara and Hashimoto (1990) from spinach PSII membranes, since the latter enzyme seemed to be a metal-type protease, insensitive to PMSF, with a neutral-alkaline pH optimum.

The susceptibility to inhibitors of serine-type proteases was used to study the involvement of EP in light- and ·OH-induced proteolysis. Isolated thylakoids were pretreated with PMSF and TLCK and then incubated in light at 80 W m^{-2}, in darkness and in darkness with Asc-Fe-H_2O_2 system. As expected, light and the ·OH-generating system promoted the degradation of many polypeptidic bands in control thylakoids (Fig. 4). However, when EP was inhibited

Table 2 Partial purification of an endopeptidase from oat thylakoids

Step	Protein (mg)	Specific activity (mg hemog h^{-1} (mg protein)$^{-1}$)	Recovery (%)	Purification (-fold)
Thylakoids	50.4	3.0	100	—
Acetone powder	2.9	42.5	66	14.2
Hemoglobin-agarose	0.1	362.6	22	120.9

Details of each step are provided in Materials and Methods.

Table 3 Influence of protease inhibitors on EP activity from oat thylakoids

Inhibitor	Concentration	Inhibition (%)
N-ethyl maleimide	1.0 mM	2
Iodoacetamide	0.5 mM	0
Pepstatin	0.1 mg ml^{-1}	0
EDTA	5.0 mM	4
o-Phenanthroline	1.0 mM	0
PMSF	1.0 mM	71
TLCK	0.1 mM	62
PMSF+TLCK	1.0+0.1 mM	82

After a 1-h incubation of the isolated enzyme with each inhibitor, EP activity was assayed under standard conditions. Control EP activity was 348 mg hemoglobin h^{-1} (mg protein)$^{-1}$. For details see Materials and Methods.

by PMSF and TLCK, the rate of breakdown of most of these polypeptides was decreased in both the case of light and Asc-Fe-H_2O_2 treatments (Fig. 4). The densitograms of polypeptide patterns shown in Figure 4 demostrate that the extent of inhibition of proteolysis varied among polypeptides, ranging from 46% for the band of 56 kDa to 82% for that of 32 kDa, but the results did not differ significantly between light- and Asc-Fe-H_2O_2-treated chloroplasts. These results indicate that light-promoted and active-oxygen-promoted proteolysis in oat chloroplasts could be catalyzed by a serine-type protease, which is active at acid pH and is associated with the thylakoids.

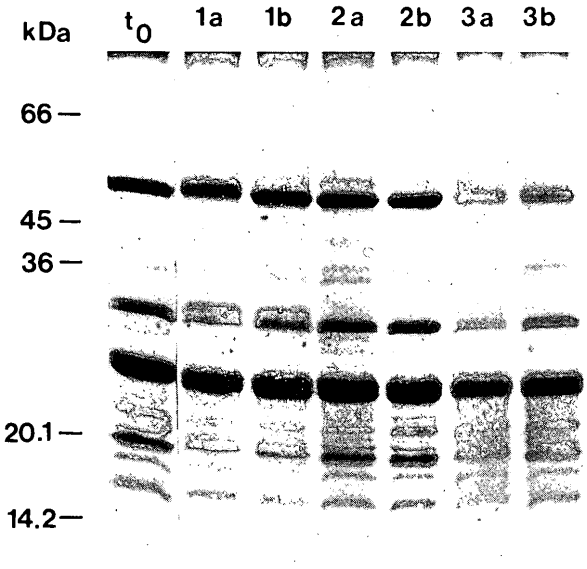

Fig. 4 Effect of inhibitors of EP on light- and 'OH-induced proteolysis in isolated oat thylakoids. Aliquots of freshly prepared thylakoids (20 μg of protein each), resuspended in 100 mM citrate-P_i buffer, pH 4.8, were preincubated without (a) or with 1 mM PMSF and 0.1 mM TLCK (b) for 1 h at 4°C. Subsequently, aliquots of each mixture were incubated for 0 h (t_0) or 2 h at 24°C, in light (80 W m^{-2}) with water (1), in darkness with water (2) or in darkness with 10.0 mM ascorbic acid–1.0 mM $FeCl_3$–10.0 mM H_2O_2 (3). Results were analyzed by SDS-PAGE.

The regulation of EP activity by oxygen radicals—The high rates of proteolysis and EP activity observed in oat chloroplasts subjected to photooxidative conditions or treatment with oxygen radicals could be due to two possible mechanisms, mediated by active oxygen species, namely, modification of the kinetic properties of EP or alterations in substrate proteins, that increase their susceptibility to attack by EP.

In order to study these possibilities we first measured the influence of the concentration of substrate (hemoglobin) on EP activity of both the isolated enzyme and thylakoids, that had previously been treated without or with H_2O_2 and Asc-Fe-H_2O_2. A typical profile of EP activity in control thylakoids is shown in Figure 5. Half-maximal activity was observed at approximately 158 μg hemoglobin ml^{-1}. In the case of the isolated enzyme, this value was lower (95 μg hemoglobin ml^{-1}), probably as consequence of higher accessibility of the hemoglobin to the active site of EP. Neither H_2O_2 nor 'OH significantly modified this behaviour of EP. Indeed, values of the "apparent K_m" of 170 and 100 μg hemoglobin ml^{-1} were found for the thylakoids and the isolated enzyme, respectively, that had been treated with H_2O_2. Values of 160 and 108 μg hemoglobin ml^{-1} were obtained with Asc-Fe-H_2O_2-treated thylakoids and the similarly treated isolated enzyme, respectively. Moreover, V_{max} values of EP activity were not significantly different among control, H_2O_2- and Asc-Fe-H_2O_2-treated preparations of thylakoids and the isolated enzyme (data

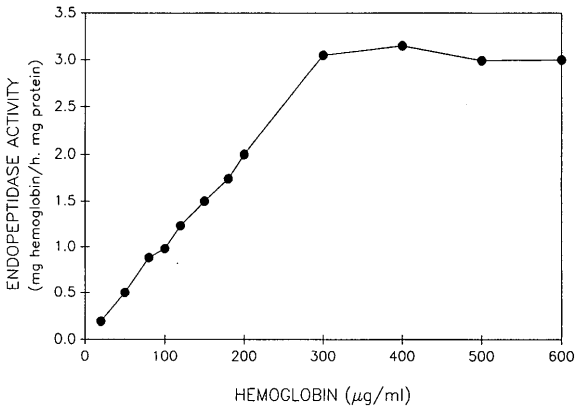

Fig. 5 Influence of the substrate concentration on the "acid" endopeptidase activity of oat thylakoids. EP activity of isolated thylakoids (5 μg of protein) was assayed at different concentrations of hemoglobin, from 0 to 1.0 mg ml^{-1} (results are only shown up to 0.6 mg hemoglobin ml^{-1}). Other assay conditions were the same to those described for the standard assay. For details see Materials and Methods.

not shown).

Since active oxygen failed to modify the kinetics properties of EP, as suggested by the results presented here, increased rates of proteolysis could have resulted from the oxidative modification of substrates which, in turn, increased their susceptibility to EP-catalyzed hydrolysis. This possibility was preliminarily examined by treating both soluble proteins and thylakoids with Asc-Fe-H_2O_2 with subsequent incubation without or with isolated EP at pH 4.5. In the case of control and Asc-Fe-H_2O_2-treated soluble proteins, the appearance of cleavage products was almost negligible in the absence of added EP (Table 4), as expected, since in oat chloroplasts all the "acid" protease activity seems to be associated with thylakoids (Casano et al. 1990). Upon addition of isolated protease, significant proteolysis was observed even in controls, but proteolysis increased with the extent of pretreatment with the Asc-Fe-H_2O_2 system. A polypeptide of 55 kDa (presumably the large subunit of RuBisCO) seemed to be one of the substrates of EP, the rate of degradation of which increased with the increases in levels of ·OH radicals (Table 4). Recently, Metha et al. (1992) reported that oxidative stress caused by high concentrations of cupric ions resulted in cross-linking of RuBisCO, translocation of the protein to thylakoid membranes, and rapid degradation of the protein. These authors proposed that the breakdown of the oxidatively altered protein might be catalyzed by a protease associated with chloroplast membranes.

Table 4 EP-catalyzed hydrolysis of ·OH-treated proteins from oat chloroplasts

Proteins	−EP	+EP
	(% relative to control)	
Soluble		
+·OH	0 (±0)	58 (±0.6)
++·OH	29 (±10)	130 (±2.5)
Thylakoid		
+·OH	37 (±1.8)	63 (±1.6)
++·OH	205 (±9.2)	455 (±2.6)
RuBisCO (LSU)		
+·OH	5 (±2.1)	19 (±1.8)
++·OH	17 (±1.6)	73 (±4.5)

Either soluble proteins or thylakoids were treated for 1 h at pH 8.0, at room temperature and in darkness with 0 mM, 0 mM and 0 mM (control); 2.0 mM, 0.2 mM and 2.0 mM (+·OH); and 10.0 mM, 1.0 mM and 10.0 mM (++·OH) of ascorbic acid, $FeCl_3$ and H_2O_2, respectively. After the pH had been lowered to 4.8, proteins were incubated without (−EP) or with (+EP) partially purified EP for 2 h at 39°C. Reactions were stopped with 4.5% (final concentration) TCA and incubation for 30 min at 0°C. Proteins were pelleted (14,500×*g*, 10 min), and products of hydrolysis were quantified in terms of increment (after 2-h incubation) in the absorvance at 280 nm of the supernatant. Proteolysis in controls was quantitated as follows: soluble −EP, 0.004; soluble +EP, 0.128; thylakoid −EP, 0.084 and thylakoid +EP, 0.146. Values (means of three independent experiments) are presented as the percent increase in A280 nm relative to the corresponding control. In other experiments, reactions with soluble proteins, treated as stated above, were stopped with SDS sample buffer and analyzed by SDS-PAGE and subsequent densitometry. Degradation of a polypeptide of 55 kDa (RuBisCO LSU) was estimated by comparing densitograms from before and after a 2-h treatment. Breakdown in controls was: −EP, below detectable level; +EP, 13% h^{-1}. Values (means of results of two independent experiments) are presented as percent increase in the degradation rate relative to the corresponding control. For details see Materials and Methods.

In thylakoids, pretreatments with the ·OH-generating system increased proteolysis even in absence of added EP since membranes contain endogenous protease(s) (Table 4). However, the addition of isolated EP promoted the hydrolysis of membrane proteins, especially if they had been oxidatively damaged by Asc-Fe-H_2O_2. The chemical nature of such oxidative modifications remains to be elucidated. On the basis of data obtained from studies with animal proteins (Davies et al. 1987, Davies and Delsignore 1987) it is reasonable to propose that some chloroplastic proteins exposed to ·OH radicals may undergo a change in their secondary and/or tertiary structure, probably as a consequence of radical reactions with tryptophan, tyrosine, histidine and/or cysteine residues.

Results from several authors (Bradley et al. 1991, Kyle et al. 1984, Krause and Cornic 1987) indicate that the rapid hydrolysis of the D1 protein of PSII in the light involves oxidative modifications of the protein which increase its rate of degradation by a serine-type protease (Virgin et al. 1991). We consider that this model can be extended to explain the increased rates of hydrolysis of several other membrane-associated or even soluble proteins of oat chloroplasts under photooxidative conditions. Modifications by ·OH radicals of these proteins and the rapid EP-catalyzed hydrolysis of the altered proteins seem to be at least two, of the key steps in this process.

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