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Ribulose-1,5-bisphosphate carboxylase/oxygenase specific proteolysis in barley chloroplasts during dark induced senescence

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Abstract

Intact chloroplasts were isolated from dark-senescing primary barley (*Hordeum vulgare* L.) leaves in order to study selective ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) degradation by the stromal and membrane fractions. RuBPCO specific degradation was estimated and characterised applying sensitive avidin-biotin ELISA method with non-modified or oxidatively modified biotinylated RuBPCO (BR) as substrates. Distinct proteolytic activities were detected. They differed in ATP and divalent metal ion dependence, protease inhibitory profile, and dynamics in the time-course of dark-induced senescence. The results supported involvement of ATP- and metal ion-dependent serine type proteolytic activity against non-modified BR early in induced senescence and appearance of ATP-independent activity at later stage. Active oxygen-modified BR was degraded by ATP-independent serine-type protease probably containing essential SH-groups and requiring divalent metal ions.

Additional key words: ATP; biotinylated RuBPCO; dry/fresh mass ratio; EDTA; ELISA; *Hordeum vulgare* L.; *p*-hydroxymercuribenzoate; Mg; phenyl-methyl sulphonyl fluoride; proteolytic activity.

Introduction

Leaf senescence involves massive mobilisation of nutrients from senescing leaves to new leaves, developing fruits, seeds, and buds thus contributing to nutrient cycling (Hortensteiner and Feller 2002). The precise triggering mechanism of senescence still remains unclear but decline in photosynthesis below a certain threshold level could be a possible initiating factor (Quirino *et al.* 2000). Chloroplast is the earliest and major target of senescence induced catabolism (Hortensteiner and Feller 2002). However, the mechanism of chloroplast protein degradation in senescence is not completely elucidated.

Proteolysis of chloroplast proteins begins in an early phase of senescence. The abundant chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO, EC 4.1.1.39), which has negligible turnover in mature leaves and is considered as a nitrogen store, undergoes an abrupt change in turnover rate early in senescence (Hortensteiner and Feller 2002). The rapid selective degradation of RuBPCO in senescing leaves has been interpreted from two points of view. One is that the vacuole is the major intracellular compartment involved in RuBPCO degradation, the other viewpoint is that RuBPCO is hydrolysed by proteolytic enzymes inside the chloroplast (Minamikawa *et al.* 2001). Multiple degradation pathways may exist for stromal proteins and vacuolar endopeptidases might also be involved under certain conditions, especially in advanced senescence (Hortensteiner and Feller 2002). As in senescing leaves the chloroplasts are progressively dismantled but remain apparently intact and are disintegrated only in the last stage (Inada *et al.* 1998), the specific degradation of RuBPCO early in senescence is most probably located inside the chloroplasts. Several proteases are up regulated in senescing leaves but they are targeted to sites outside the chloroplast, accumulate in advanced senescence, and their contribution to the degradation of proteins inside the chloroplast is unclear (Pinedo *et al.* 1996, Distefano *et al.* 1997, Minamikawa *et al.* 2001). Plastids possess their own proteolytic systems within each of the major chloroplast compartments: the ATP-dependent Clp and FtsH proteases in the stroma and stroma-exposed thylakoid membranes, respectively, the ATP-independent DegP

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Abbreviations: AO – active oxygen; AO-BR – active oxygen-modified biotinylated RuBPCO; ATP – adenosine-5′-triphosphate; BR – biotinylated RuBPCO; DM – dry mass; EDTA – ethylene diamine tetraacetic acid; ELISA – enzyme-linked immunosorbent assay; FM – fresh mass; PBS – 20 mM phosphate buffered saline, pH 7.2; PHMB – *p*-hydroxymercuribenzoate; PMSF – phenyl-methyl sulphonyl fluoride; TBS – 50 mM Tris, 150 mM NaCl, pH 8,0.
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proteases within the thylakoid lumen and on both sides of thylakoid membranes, and the SppA protease on the stromal side of the thylakoid, all of them homologous to proteases well characterised in bacteria (Adam and Clarke 2002). However, their endogenous substrates, physiological relevance, and regulation are still not well understood. No senescence-specific *de novo* synthesis of chloroplast proteases has been clearly demonstrated to date. RuBPCO proteolysis by zinc-containing metalloprotease increases in chloroplasts from *Pisum sativum* leaves subjected to nitrogen starvation-induced senescence (Roulin and Feller 1998). RuBPCO aggregates and associates with thylakoid membranes under oxidative stress and oxidation of RuBPCO may be a necessary step triggering its degradation in senescence (Mehta *et al.* 1992, Desimone *et al.* 1998). Non-enzymatic RuBPCO breakdown by AO in advanced senescence could not be excluded as well (Ishida *et al.* 1997). AO species are involved as signals in the induction phase of the programmed cell death (the final stage of the senescence program) and as participants in the terminal destruction phase (Jabs 1999). The oxidative modification of purified RuBPCO increased its susceptibility to digestion by ATP-dependent chloroplast protease not belonging to the Clp protease complex (Desimone *et al.* 1998). However, it is not known if the same is observed in dark-induced senescence, which is not an intrinsically oxidative treatment. Whether or not ATP is required and if RuBPCO is modified (*e.g.* by reactive oxygen species) prior its degradation in senescence are questions still under debate (Hortensteiner and Feller 2002).

The aim of the present investigation was to compare and characterise the selective degradation of oxidatively modified and not-modified RuBPCO in chloroplast fractions from dark-senescing primary barley leaves.

Materials and methods

Plants and dark treatment: Barley seedlings (*Hordeum vulgare* L. cv. Obzor) were grown under 12/12 h photoperiod, 27/22 °C, and 63 W m^{-2} irradiance in Huffaker's nutrient solution as described by Metodiev and Demirevska-Kepova (1992). Placing intact 10-d old seedlings in continuous darkness for various periods induced senescence symptoms. Control plants were kept in normal day/night cycle. All analyses were performed on the first leaf, which was fully expanded at the beginning of the treatment.

Leaf extracts, protein, RuBPCO, and pigment determinations: Two grams of leaf material were homogenised at 4 °C with 8 cm^3 of ice-cold 100 mM Tris-HCl, pH 8.0 containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA (disodium salt), 2 mM PMSF, 12,5 % glycerol (v/v), 20 mM β-mercaptoethanol, and 120 mg *Polyclar AT*. After centrifugation at 15 000×*g* for 30 min, the supernatant was used for estimation of total soluble protein by the method of Bradford (1976) and of RuBPCO content by ELISA method (Metodiev and Demirevska-Kepova 1992). Leaf pigment amounts were determined according to Arnon (1949) and calculated using the formula of Mackinney (1941).

Chloroplast isolation: Seedlings were kept in darkness for at least 24 h prior to the isolation of chloroplasts in order to exhaust the starch reserves. All the procedures were carried out at 0-4 °C. Chloroplasts were prepared according to the method of Nakatani and Barber (1977) with modifications (Demirevska-Kepova *et al.* 1989). The integrity of chloroplasts, estimated by the latency of the ferricyanide photoreduction (Walker *et al.* 1987), was on average 88±4 %. Chloroplasts were osmotically ruptured by re-suspension in ice-cold distilled water, buffered with 50 mM Tris-HCl, pH 8.0 containing 5 % (v/v) glycerol, incubated in ice for one hour, and centrifuged at 12 000×*g* for 30 min in order to separate stromal supernatant and membrane pellet. Contamination by other subcellular compartments was below 0.5 % for acid phosphatase (vacuolar marker) and below 0.4-0.7 % for catalase (peroxisomal marker). Acid phosphatase activity did not vary by increasing the duration of the dark treatment, whereas catalase activity was higher in advanced senescence.

RuBPCO-specific proteolytic activity was assayed by means of modified avidin-biotin ELISA method (Simova-Stoilova *et al.* 2000) at pH 8 (chosen to be physiologically relevant for the chloroplast stroma). Barley RuBPCO was purified as previously described (Demirevska-Kepova and Simova 1989). Biotinylation was performed in 200 mM borate buffer, pH 9.0, by incubation of purified RuBPCO with N-hydroxysuccin-imidobiotin in a molar ratio of 1 : 100 for 3 h at room temperature. Additional oxidative modification was carried out according to Desimone *et al.* (1998). Incubations for RuBPCO degrading activity in the stromal fractions were performed on microtitre plates, which had been blocked with 10 kg m^{-3} ovalbumin, washed, and dried. The incubation mixtures contained (final concentrations in a volume of 0.36 cm^3): stromal fraction 0.2 kg m^{-3} protein; 5 kg m^{-3} ovalbumin; TBS or TBS supplemented with 5 mM $MgCl_2$, or TBS supplemented with 5 mM $MgCl_2$ plus 5 mM ATP (in the analysis of ATP and metal ion dependence); as protease inhibitors 150 μM PHMB, 10 mM EDTA and 2.5 mM PMSF; 10 g m^{-3} BR or AO-BR. The protease inhibitors were added from concentrated stocks separately at the beginning of inhibitory analysis or as stop reagents at the end. The reaction was started with the substrate BR or AO-BR, allowed to proceed for one hour at 30 °C, and stopped with protease inhibitors. Thereafter,

the quantity of non-degraded BR or AO-BR was estimated in sandwich ELISA using immobilised avidin and avidin-peroxidase conjugate as described by Simova-Stoilova *et al.* (2000). The standard curves were prepared using a range of BR or AO-BR concentrations from 1 to 10 g m^{-3} (0.1 cm^3 per well) in TBS with 5 kg m^{-3} ovalbumin, and protease inhibitors. In the analyses of the thylakoid membrane fractions, incubations for proteolytic activity were made in Eppendorf microcentrifuge tubes instead of microtitre plates (final chlorophyll concentration of 0.25 kg m^{-3}) and the samples were centrifuged to pellet the membranes prior to their transfer to the avidin plate. $ZnCl_2$ was added in final concentration of 1 mM.

Results and discussion

Time-course of senescence symptoms in dark-treated mature primary barley leaves: Dark-induced senescence is often used as a reproducible experimental system to induce uniform senescence symptoms in the leaves, based on carbon-starvation related nutrient re-mobilisation (Brouquisse *et al.* 1998). The first leaf of intact barley seedlings entered the phase of full expansion by the day eight after sowing. The phase of slow natural senescence provoked by the development of the second leaf began after the day twelfth (Simova-Stoilova *et al.* 2000). Time-course changes in leaf pigment and protein contents (parameters indicative for senescence progression) are presented in Fig. 1. Statistically significant difference in chlorophyll content ($p < 0.01$, Fig. 1*A*) and total protein ($p < 0.001$, Fig. 1*B*) appeared at 2nd day of the dark treatment. The lack of significant change in these parameters at 1st day allowed us to assume that plants kept 24 h in darkness could serve as a control in analyses on chloroplast preparations. No differences in chlorophyll *a/b* ratio were observed between controls and dark treated plants. Content of carotenoids remained constant (Fig. 1*A*). DM/FM ratio of the first leaves in controls was rather constant, whereas in dark-senescing leaves small, but steady diminution of this ratio was observed, reflecting re-mobilisation of components (Table 1). Decline in RuBPCO quantity was observed during both natural ageing and dark treatment. Decrease in RuBPCO was accelerated by irradiance deprivation (Table 1) in support of the selective RuBPCO loss during dark induced senescence.

Table 1. Dry/fresh mass (DM/FM) ratio and RuBPCO content as a percent of total soluble protein in the leaves of control and dark-treated barley seedlings. Means of three replicates. SE was less than 10 %.

Time after sowing [d]	DM/FM		RuBPCO content [%]	
	Control	Dark treatment	Control	Dark treatment
11	0.1170	0.1120	47.5	47.5
13	0.1248	0.1073	30.0	27.0
15	0.1228	0.0895	17.5	7.5

RuBPCO degrading activity in the sub-chloroplastic fractions: Our preliminary analysis of stromal and membrane chloroplast fractions using SDS-PAGE in the presence and in the absence of β-mercaptoethanol as described by Mehta *et al.* (1992) revealed some RuBPCO aggregation but no association of the enzyme to the membrane fraction (values not presented). This implies that during dark-induced senescence RuBPCO must be degraded in the stroma without translocation to the thylakoid membranes. However, it is not excluded that RuBPCO degrading protease(s) could be membrane-linked. For example, the Zn-and-ATP-dependent FtsH protease, localised in the thylakoid membrane with ATP binding and catalytic sites protruding in the stroma, could degrade membrane as well as stroma proteins (Lindahl *et al.* 2000). For that reason, RuBPCO degrading activity was investigated separately in the stromal and membrane fractions.

The specific RuBPCO-degrading activities in the stromal fraction of chloroplasts obtained after 24 (control), 48 (early stage of senescence), and 96 h (advanced stage) of dark treatment using AO-BR or BR as substrates were compared and the dependence on ATP and Mg^{2+} was followed (Fig. 2). As the reaction mixture contained ovalbumin in a fairly high concentration (about 500 times greater than that of BR), most probably the test system reflected selective RuBPCO degradation. In the control and early stage BR was practically not degraded in the absence of ATP and Mg^{2+} (Fig. 2*A*). Small but significant increase in ATP- and Mg^{2+}-dependent proteolytic activity against BR was observed in the early stage of senescence (*striped and squared columns*), whereas a novel activity appeared later, which was independent of ATP and Mg^{2+} (*white columns*). Proteolysis of AO-BR in the control was observed even in the absence of ATP and Mg^{2+} (Fig. 2*B*, *white columns*). Divalent ions and ATP had no stimulating effect on the degradation of AO-BR (*squared and striped columns*). The Mg^{2+}-dependent proteolytic activity against AO-BR slightly increased in the early stage and decreased thereafter. Inhibitor effects of PHMB, PMSF, and EDTA on RuBPCO proteolysis (tested in buffer supplemented with ATP and Mg^{2+}) are presented

in Fig. 3. In the case of BR, proteolytic activity was sensitive to inhibitors for serine- (PMSF) and metallo- (EDTA) proteases, whereas the thiol-protease specific inhibitor PHMB caused only slight inhibition (Fig. 3*A*). Surprisingly, the inhibitory effect of the tested substances decreased with the progress of senescence. Possible explanations could be change in the protease pattern (induction of proteolytic activities toward which the small number of tested inhibitors was not effective) or some non-enzymatic RuBPCO degradation by AO in advanced senescence. In the case of AO-BR, the inhibitor sensitivity profile indicated the persistence of protease activity sensitive to the serine type protease inhibitor PMSF (Fig. 3*B*). Strong inhibition by the metal ion chelator EDTA was also observed, which diminished with the progress of senescence. The inhibitor of cysteine type proteases

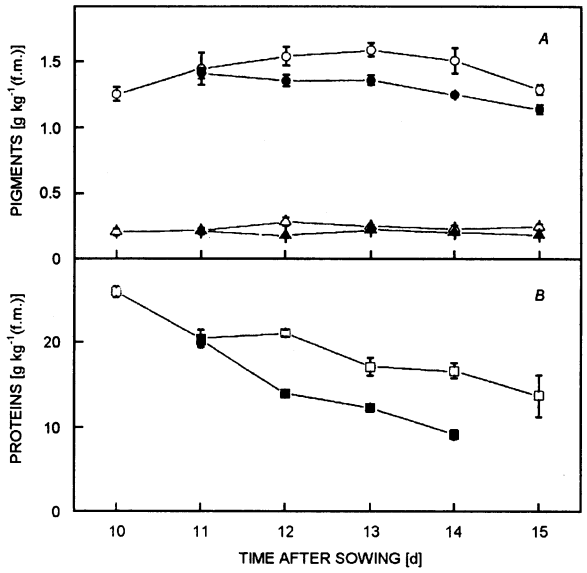

Fig. 1. Time course of leaf pigment content (*A*) and total soluble protein (*B*) in control (*open symbols*) and dark-treated (*filled symbols*) barley primary leaves on FM basis. Dark treatment began 10 d after sowing. *Circles* – chlorophyll (*a*+*b*); *triangles* – carotenoids; *squares* – total soluble protein. Means ± SE of three replicates.

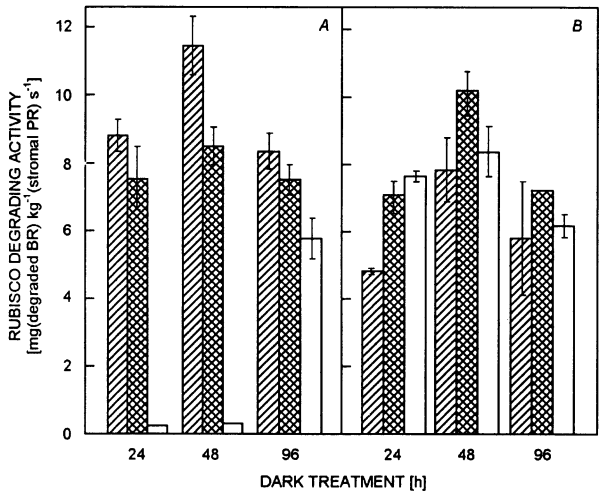

Fig. 2. Effect of ATP (*striped columns*) and Mg^{2+} (*squared columns*) on the RuBPCO-degrading activity in the stromal fraction of chloroplasts isolated after 24 (control), 48 (early stage), and 96 (advanced stage) h of induced senescence, respectively. *White columns* – activity without ATP and Mg^{2+}. *A* – non-modified BR as a substrate, *B* – AO modified BR as a substrate. Means ± SE of three replicates.

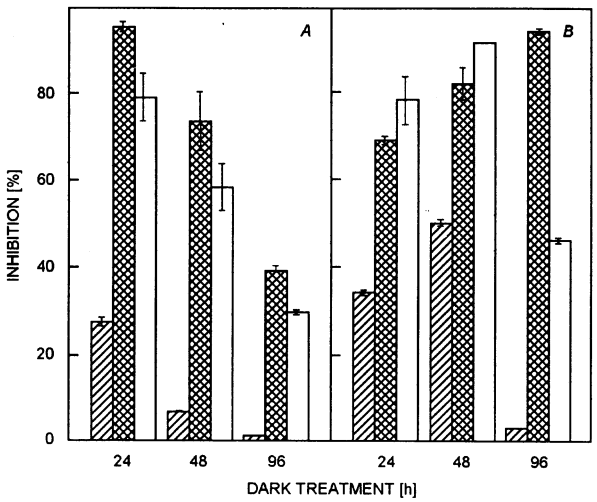

Fig. 3. Effect of protease inhibitors on the RuBPCO-degrading activities in the stromal fractions of chloroplasts isolated after 24, 48, and 96 h of dark treatment. *A* – non-modified BR as a substrate, *B* – AO modified BR as a substrate. *Striped columns* – inhibition by PHMB; *squared columns* – inhibition by PMSF; *white columns* – inhibition by EDTA. Means ± SE of three replicates.

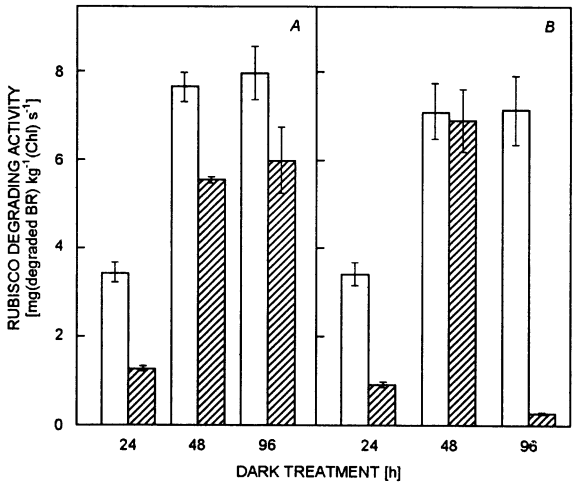

Fig. 4. RuBPCO degradation by thylakoid-bound proteases from chloroplasts isolated after 24, 48, and 96 h of dark treatment. *White columns* – effect of ATP in the presence of Zn^{2+}, *striped columns* – activity without ATP. *A* – non-modified BR as a substrate, *B* – AO modified BR as a substrate. Means ± SE of three replicates.

(PHMB) was more effective to modified than non-modified BR as a substrate, indicating that essential SH-groups might be involved in proteolysis of AO-BR, but this effect disappeared later in senescence. These results indicate the complex nature of the specific proteolytic activity against RuBPCO and the coexistence probably of several proteases. Our results support the participation of metalloprotease activity against RuBPCO in induced senescence, described by Roulin and Feller (1998). However, the results differ from that obtained by Desimone *et al.* (1998) about ATP-dependent non-serine type proteolytic activity against AO-modified radioactively labelled RuBPCO in chloroplasts, which could be due to the different mode of labelling. This difference rises the question about the importance of substrate modifications in RuBPCO selective proteolysis and about the reliability of labelled RuBPCO as a substrate reflecting its endogenous degradation; common problem in any labelling.

The results concerning RuBPCO degradation by thylakoid-bound activity are presented in Fig. 4. Proteolytic activity against BR was clearly stimulated by ATP in the control and some persistent increase was observed during dark-induced senescence. The ATP-independent proteolytic activity against AO-BR increased early in senescence and decreased later (Fig. 4*B*). The inhibitory analysis revealed serine type protease activity against non-modified BR (93 % of inhibition by PMSF) and serine and metalloprotease type activity against AO-BR (29.0 % of inhibition by EDTA and 33.4 % of inhibition by PMSF) in the controls. The sensitivity to these inhibitors diminished with senescence progression. According to Lindahl *et al.* (2000) mild trypsin treatment (1 g m^{-3} trypsin for 20 min in ice bath) of thylakoid membranes completely abolishes FtsH protease activity. However, such treatment only partially decreased the membrane-linked RuBPCO degrading activity (values not presented), so this activity was most probably different from FtsH protease.

In conclusion, distinct proteolytic activities were detected degrading both BR and AO-BR as substrates, differing in ATP and divalent metal ion dependence, protease inhibitory profile, and dynamics in the time-course of dark-induced senescence. However, the general increase of RuBPCO proteolytic activity in chloroplasts during dark induced senescence was rather small, suggesting that some kind of protein modification may trigger RuBPCO degradation. AO-modification of RuBPCO may be the most probable factor that destabilises RuBPCO in senescence (Desimone *et al.* 1998). However, it seems that senescing leaves retain, at least in part, their defence potential against AO species (Merzlyak and Hendry 1994). The absence of RuBPCO associated with membrane fractions indirectly indicates conserved capacity of dark senescing chloroplasts to detoxify AO species. A hypothetical mechanism of redox regulation of RuBPCO turnover was presented by Moreno *et al.* (1995) in which the oxidation of critical SH groups may be driven under a mildly reducing environment, however, this hypothesis still needs substantial experimental support. The participation of some of the bacterial-like chloroplast proteases in RuBPCO degradation during senescence remains to be elucidated.

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