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The activity of the 20S proteasome is maintained in detached wheat leaves during senescence in darkness

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Abstract

In the present paper, we studied the participation of the 20S proteasome, the proteolytic component of the ubiquitin–proteasome pathway, in the remobilization of bulk proteins in senescing wheat leaves. The detached leaves of 15-d-old plants were incubated in darkness for several days, and various proteolytic activities were analysed in soluble extracts prepared at 0, 48 and 96 h after detachment. The endoproteolytic activity, measured at pH 7.5 and 5.4, increased more than 10-fold and the total peptidasic activity increased up to 5-fold after 96 h of incubation in the dark, when expressed as specific activity. In the same period, the leaf-protein content decreased to less than 50% of that present at the initial time. The 20S proteasome chymotrypsin-like activity remained constant when it was expressed as activity per leaf fresh weight and resulted 2-fold higher in terms of specific activity. The western blot analysis showed that the amount of 20S proteasome protein and ubiquitin–protein conjugates also remained constant until 4 d of incubation in darkness. These results indicate that the ubiquitin–proteasome pathway remains functional until the late phases of senescence suggesting that it may participate in the regulatory aspects of the process rather than in the massive protein breakdown.

Keywords: 20S proteasome; Proteolysis; Senescence; Ubiquitin; Wheat

1. Introduction

Senescence is the final stage of leaf development leading to the death of the organ [11]. It is a genetically programmed process [26], involving leaf protein and chlorophyll degradation. Membrane integrity and the subcellular compartmentation are maintained until the latest phase, allowing for a proper cooperation between various organelles involved in the catabolism of leaf constituents [11].

Protein degradation during senescence involves the induction of several proteolytic activities [32] that contribute to the degradation of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39), the most abundant protein in leaves, which is massively hydrolysed during the first stages of leaf senescence together with other proteins [11].

The ubiquitin–proteasome proteolytic system is responsible for the specific degradation of abnormal, short-lived and regulatory proteins in the cytoplasm and nucleus of the eukaryotic cells [20,22]. In addition, this pathway has been shown to be involved in the degradation of the bulk of proteins in the insect and mammalian cells under certain metabolic conditions [19,28].

In higher plants, the ubiquitin–proteasome components have been isolated and characterised [3,32–34]; this proteolytic system has also been shown to participate in cell-cycle progression [16], degradation of short-lived regulatory proteins [8], elicitation of defence responses [2], auxin response, photomorphogenesis and pollen germination [29] (for a review see [7,10,32,33]).

The involvement of the ubiquitin–proteasome pathway in senescence is suggested by biochemical analysis and evidence of the expression of genes coding for components of the ubiquitin pathway [9,15,17,27,31]. On the other hand,

Abbreviations: Cbz, benzyloxycarbonyl ; DEAE, diethylaminoethyl ; DTT, dithiothreitol ; NEM, N-ethyl-maleimide ; pNA, p-nitroanilide ; PVDF, polyvinylidene difluoride ; Rubisco, ribulose-1,5-bisphosphate carboxylase ; oxygenase ; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis ; TCA, trichloroacetic acid

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studies on the expression of an α-type 20S proteasome subunit gene provided no support for a role of the proteasome proteolytic pathway during the senescence process, at least in massive protein degradation [1].

In order to clarify this point, we investigated several proteolytic activities in senescing wheat leaves as well as possible changes in the 20S proteasome proteolytic activity. Our results suggest that the induction of proteolytic activities different from that of the 20S proteasome can account for the massive protein degradation observed. The fact that the 20S proteasome, as well as the ubiquitin conjugates, is kept at a constant level throughout the senescence process indicates that the ubiquitin–proteasome pathway remains functional until the late phases of senescence, suggesting its participation in the regulatory aspects of the process rather than in the bulk degradation of proteins.

2. Results

When excised leaves of 15-d-old wheat plants were incubated in the dark for several days, there was a fast induction of various proteolytic activities. After 96 h in the dark, the endoproteolytic activity increased nearly 400% when expressed as activity per leaf fresh weight (Fig. 1A), and as much as 1000% when expressed as specific activity (Fig. 1B). The total peptidasic activity increased more than 200% on a fresh-weight basis, and up to 500% when expressed as specific activity (Fig. 1A, B). During this period, the leaf-protein concentration decreased to less than half of the initial concentration (Fig. 1A). The 20S proteasome activity remained constant on a fresh-weight basis during the whole dark period, and only doubled its specific activity in the same period (Fig. 1A, B). In order to further support these results, the extracts obtained from fresh leaves and from 96 h dark-induced leaves, containing the same amount of soluble protein, were fractionated by ion exchange chromatography and analysed for Cbz–GGL–pNA and azocasein-hydrolysing activities. As can be seen in Fig. 2A and C, one symmetrical peak of chymotrypsin-like activity, eluting at the same sodium chloride concentration, was detected in the eluates from extracts obtained from fresh or dark-induced leaves. Remarkably, the total activity recovered from the dark-induced leaf extract doubled that obtained from the leaf extracts prepared from the non-induced leaves (Fig. 2A, C). Fractions were also analysed by western blotting using a polyclonal antiserum raised against maize 20S proteasome. The fractions containing immunoreactive proteins were coincident with those exhibiting enzymatic activity in both senescing (Fig. 2D) and non-senescing leaf samples (Fig. 2B).

Fig. 2A and C also shows the profile of azocasein-hydrolysing activity under both conditions (fresh and dark-induced leaf extracts). As can be seen, a broad peak, eluting ahead of the 20S proteasome activity, was observed in both cases. However, a second peak of endoprotease activity,

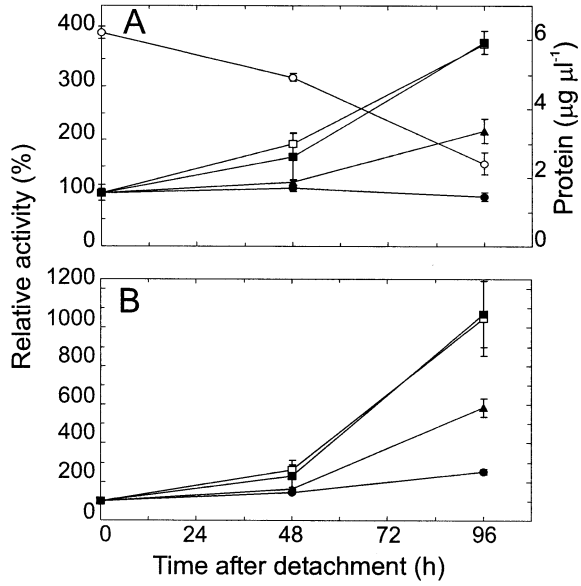

Fig. 1. Changes in proteolytic activities, during the dark-induced senescence in the detached wheat leaves. After 0, 48 and 96 h of incubation in darkness, leaf extracts were prepared and enzymatic activities were measured as described in the Methods. Activities are expressed per gram of leaf fresh weight (**A**) or per milligram of total protein (**B**). Endoproteinase activity using azocasein as substrate was assayed at pH 7.5 (□) and pH 5.4 (■), release of α-amino groups by autodigestion of extracts at pH 5.4 was detected with the ninhydrin reagent (▲) and 20S proteasome activity was determined by hydrolysis of the synthetic peptide Cbz–GGL–pNA (●). Protein content of extracts is also shown (○). Data are presented as relative values ± SEM of three replicates, with the data for 0 h after detachment set at 100. **A**, initial values are 55.5 and 55.8 UE h^{-1} g^{-1} for azocaseinolytic activity at pH 7.5 and 5.4, respectively; 7.18×10^{-12} kat g^{-1} for 20S proteasome activity; 3.66×10^{-10} kat g^{-1} and 6.2 μg $μl^{-1}$ for soluble protein concentration. **B**, initial values are 3.2 UE h^{-1} mg^{-1} for azocaseinolytic activity at both pHs; 4.22×10^{-13} kat mg^{-1} for 20S proteasome activity and 2.15×10^{-11} kat mg^{-1}.

eluting with the void volume was present only in extracts derived from the dark-induced leaves. These activities are now under study.

Western blot analysis of leaf extracts during the incubation period in the dark showed that the amount of 20S proteasome protein did not change as judged by the intensity of the main proteasomal component recognised by antibodies directed against maize (Fig. 3A) or *Candida albicans* 20S proteasome (Fig. 3B). The amount of ubiquitin complexes, detected with a specific antibody, appearing as proteins of molecular masses above 100 kDa did not vary significantly during the 96 h darkness incubation (Fig. 4).

In young wheat leaves, more than 50% of soluble protein can be accounted for by Rubisco. The SDS-PAGE pattern of the total leaf proteins during the dark incubation period showed a decrease in the intensity of all polypeptide bands. The degradation of a band near the 47.5 kDa marker, probably corresponding to the large subunit of the Rubisco (Fig. 5), was the most significant.

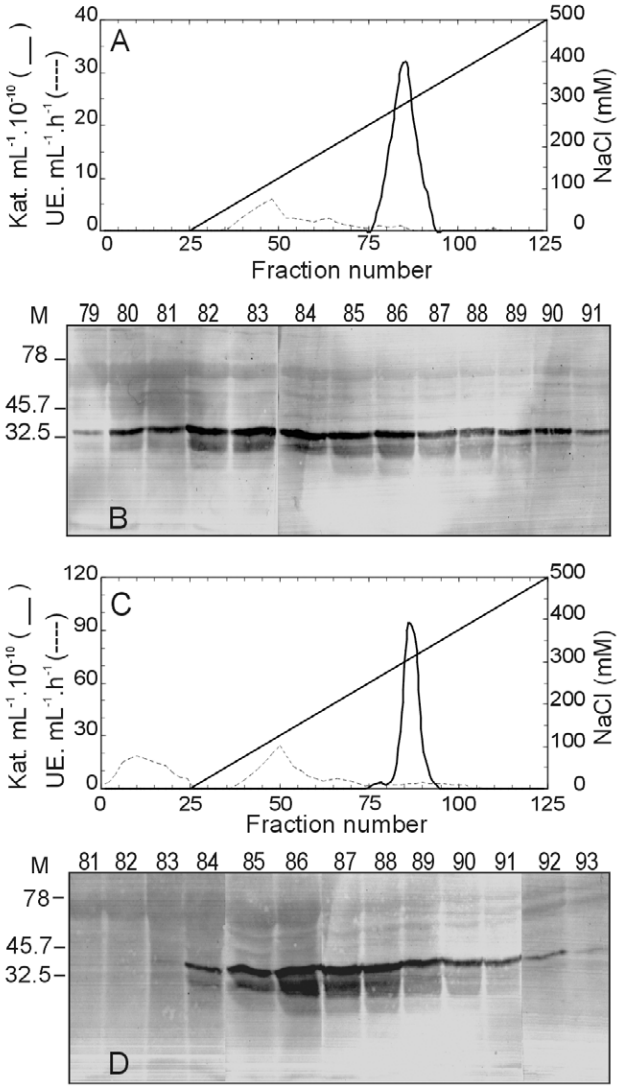

Fig. 2. The DEAE-Sephacel chromatography analysis. Senescence was induced in the detached leaves by incubation in continuous darkness. Protein extracts were prepared from 96-h-induced and non-induced leaves and equal amounts of total protein (30 mg) were analysed by DEAE-Sephacel chromatography. Fractions from non-induced (**A, B**) and induced (**C, D**) leaves were assayed for the presence of chymotrypsin-like activity (kat ml^{-1}) using Cbz–GGL–pNA as substrate (—) and azocaseinolytic activity (UE ml^{-1} h^{-1}) (----) (**A, C**) and for 20S proteasome concentration by the western blot analysis using an antimaize 20S proteasome polyclonal antibody (**B, D**). In **B** and **D**, numbers above the lanes correspond to fraction numbers of the column. M: position of molecular mass markers.

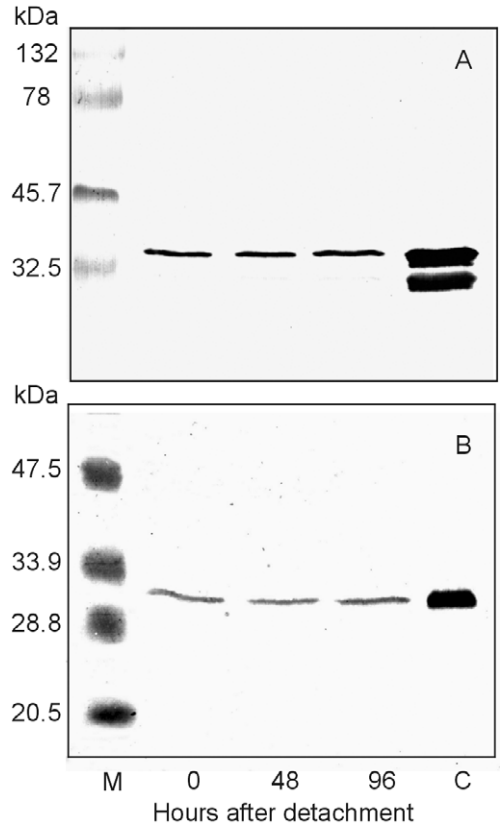

Fig. 3. The western blot analysis of the 20S proteasome in protein extracts from the detached wheat leaves incubated in continuous darkness. Protein extracts were prepared from the detached leaves after 0, 48 and 96 h of incubation in darkness. Equal amounts (3.7 mg fresh weight in **A** and 50 μg soluble protein in **B**) of each sample were separated by 15% SDS-PAGE and electrophoretically transferred to a PVDF membrane. The proteins were blotted with a polyclonal antibody against maize (**A**) or *C. albicans* (**B**) 20S proteasome. Lane C in both figures corresponds to a purified fraction of wheat 20S proteasome (3 μg protein). M: position of molecular mass markers.

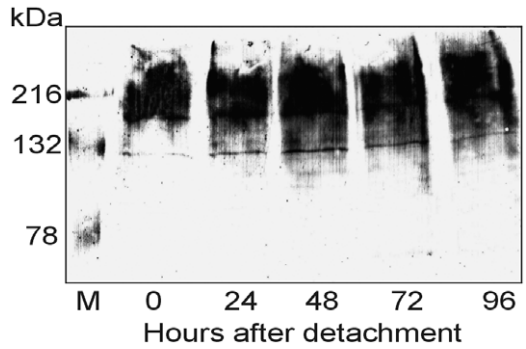

Fig. 4. The western blot analysis of ubiquitin–protein conjugates in protein extracts from the detached wheat leaves incubated in continuous darkness. Protein extracts were prepared in the presence of protease inhibitors (5 mM NEM, 2 mM EDTA, 10 μg ml^{-1} pepstatin, 10 μg ml^{-1} leupeptin) from the detached leaves after 0, 24, 48, 72 and 96 h of incubation in darkness. Equal amounts of samples (18.7 mg fresh weight) were separated on a 10% SDS-PAGE and the separated proteins were transferred to PVDF membranes. The membranes were probed with a monoclonal antibody (clone FK2) directed to ubiquitin–protein conjugates. M: position of molecular mass markers.

3. Discussion

The senescence induced by darkness has largely been used as a model for studying the mechanisms of leaf senescence [33]. However, neither the regulation of this process, involving the expression of specific genes and the induction of many proteolytic activities leading to the cell disorganisation and death nor the role of the various proteolytic activities induced has completely been understood [11].

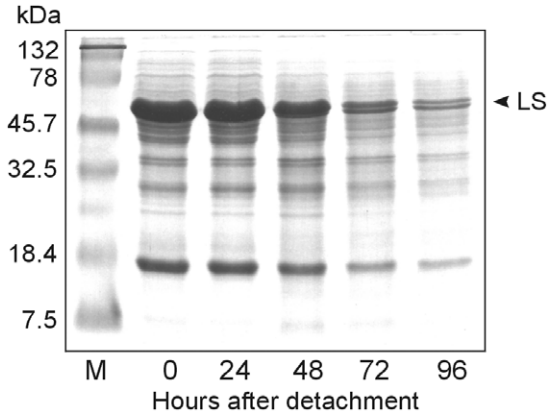

Fig. 5. The changes in soluble protein profiles of the dark-induced senescing wheat leaves. Protein extracts were prepared from the detached leaves after 0, 24, 48, 72 and 96 h of incubation in darkness. Equal amounts (3.7 mg fresh weight) of each sample were separated on a 15% SDS-PAGE and the gel was stained with Coomassie Brillant Blue R-250. LS indicates the Rubisco large subunit. M: position of molecular mass markers.

The present results show a several-fold increase in the various proteolytic activities during the dark-incubation period, and a decrease in the soluble protein content. These observations are in agreement with previous reports showing a similar pattern of protease induction accompanied by a massive protein breakdown during leaf senescence [12,14,33]. The mechanism by which Rubisco is degraded is still not clear. Several lines of evidence indicate that at least initial steps of protein degradation occur within the intact chloroplast, although an auxiliary role of the vacuole cannot be ruled out [11].

Our results show that, during the 96 h in the dark, the chymotrypsin-like activity of the 20S proteasome as well as its protein concentration was not affected (Figs 2 and 3); the same holds for the amount of ubiquitin complexes, which remained high until late in senescence (Fig. 4). These findings, which suggest that the 20S proteasome is not directly involved in the massive degradation of proteins during leaf senescence, are in accordance with those of Brouquisse et al. [6] who did not detect any induction of the 20S proteasome protein components under the dark-induced proteolysis of whole maize plants. Also, Bahrami and Gray [1] reported that in tobacco leaves and flowers, the levels of mRNA coding for one of the 20S proteasome α subunits increase in young dividing tissues, but decline during senescence in leaves and flowers, indicating a potential role of proteasome in the regulation of developmental events rather than in the bulk degradation and recycling of proteins during senescence.

The several reports showing an increase in transcripts of certain components of the ubiquitin pathway, an increase in the ubiquitin-conjugating activity and changes in the ubiquitination pattern in senescent plant tissues [9,15,17,27,31] can be interpreted by assuming that the proteolytic component of the system, the 20S proteasome, is not limiting under these conditions and/or that the ubiquitination machinery is also directed to fulfilling a different function within the cell not related to the proteasome-dependent proteolysis [20,22–24]. Our results, showing the maintenance of constant levels of the 20S proteasome as well as ubiquitinated proteins, argue in favour of a subtle regulatory role of this pathway through the specific degradation of proteins during the whole senescence process. The identification of ubiquitin–proteasome substrates along this process will contribute to a better understanding of the participation of this proteolytic system in senescing leaves. This work is now in progress in our laboratory.

4. Methods

4.1. Plant material

Wheat seeds (*Triticum aestivum* var. Pro INTA Isla Verde) were sown in 20-cm plastic pots using vermiculite as substrate and watered daily with Hoagland's nutrient solution (10 mM KNO_3) [21]. Plants were maintained in a growth chamber at 23 °C, with an irradiance of 350 μmol m^{-2} s^{-1} and a 16-h light period. Fifteen days after sowing, leaves were detached, placed in plastic boxes containing 100 ml of distilled water and incubated in darkness for 24, 48, 72 or 96 h in order to induce senescence. Control samples corresponded to the leaves detached and were immediately processed (0 h).

4.2. Preparation of cell-free extracts

At the end of each incubation period, the leaves were frozen in liquid nitrogen, ground to a fine powder and extracted with cold extraction medium (2 ml g^{-1} fresh weight) consisting of 50 mM Tris–HCl buffer (pH 7.5), 1 mM DTT and 10% (v/v) glycerol. For the analysis of ubiquitin–protein conjugates, protease inhibitors (5 mM NEM, 2 mM EDTA, 10 μg ml^{-1} pepstatin and 10 μg ml^{-1} leupeptin) were included in the extraction buffer in order to stabilise protein–ubiquitin conjugates. The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 100 000 *g* for 1 h. The supernatant obtained was desalted through a Sephadex G-25 gel-filtration column (Amersham Pharmacia Biotech, Uppsala, Sweden), frozen at –20 °C in aliquots, and used for the assays described below.

4.3. Measurement of enzymatic activities

The endoproteinase activity was assayed at pH 7.5 and 5.4 using an excess of azocasein as substrate. The reaction mixture (final volume 300 μl) contained an appropriate volume of the enzyme preparation (300 μg of soluble protein), 0.1% (v/v) β-mercaptoethanol, 5 mg ml^{-1} azocasein and 50 mM Tris–HCl buffer (pH 7.5) or 50 mM sodium acetate buffer (pH 5.4). Samples were incubated for 3 h at 37 °C and the reaction was stopped by adding 1.2 ml of 5% (w/v) TCA. After standing on ice for 15 min, the

precipitate was removed by centrifugation at 16 000 *g* for 10 min and acid-soluble products were determined spectrophotometrically at 340 nm. One unit of azocaseinolytic activity was defined as the amount of protein causing a 0.01 increase in A_{340} over the 0 time values (the reaction stopped immediately after starting). Chymotrypsinlike activity of the 20S proteasome was tested by hydrolysis of the synthetic peptide Cbz–GGL–pNA according to Bratton and Marshall [5] as modified by Goldbarg and Rutemburg [18], which is a reliable measure of the 20S proteasome activity. The release of α-amino groups by autodigestion of the extracts was determined with the ninhydrin reagent; 200 μl of an appropriate dilution of the samples (400 μg of soluble protein) were incubated at 37 °C for 1 h with 200 μl of 200 mM Tris–HCl buffer (pH 7.5). The reaction was stopped with 400 μl of 5% TCA. After standing in ice for 10 min, samples were centrifuged at 16 000 *g* for 10 min and 250 μl aliquots of the supernatant was taken for the determination of α-amino nitrogen by the ninhydrin method [36].

4.4. SDS-PAGE and immunoblotting

Protein extracts were analysed by 10 or 15% SDS-PAGE according to Laemmli [25]. For the visualisation of protein bands, the gels were stained with Coomassie Brillant Blue R-250. For the western blot analysis, proteins were transferred to PVDF membranes (Immobilon, Millipore), essentially as described by Towbin et al. [30]. Immunodetection of wheat 20S proteasome was achieved by incubating the membrane with a polyclonal antiserum raised against *C. albicans* 20S proteasome [13] or an antimaize 20S proteasome antibody kindly supplied by Dr. R. Brouquisse [6]. The ubiquitinated proteins were detected using a monoclonal antibody directed against the ubiquitin–protein conjugates, clone FK2 (Affinity Research Products, Ltd). The bound antibodies were detected with the corresponding biotinylated antirabbit (20S proteasome) or antimouse (ubiquitinated proteins) antibody and avidin-biotinylated horseradish peroxidase, using 3,3'-diaminobenzidine and H_2O_2 as substrates.

4.5. DEAE-Sephacel chromatography

Equal amounts of total protein (30 mg) of extracts from induced (96 h) and non-induced (0 h) senescing leaves were loaded on a DEAE–Sephacel column (Sigma) equilibrated with the extraction buffer. The column (1×4.5 cm) was washed with three volumes of extraction buffer and then eluted with 40 ml of a linear gradient of NaCl (0–500 mM) made in the same buffer. Fractions of 400 μl were collected and aliquots of 200 μl of each fraction were assayed for the presence of chymotrypsin-like activity using Cbz–G-GL–pNA as substrate. Proteins contained in 100 μl samples of eluted fractions were precipitated by the methanol–chloroform method [35] and separated on a 15% SDS-PAGE. The solved proteins were electrophoretically transferred to PVDF membranes and revealed with the anti-maize 20S proteasome polyclonal antibody as described above.

4.6. Protein determination

The protein content of the leaf extracts was determined according to Bradford [4] using bovine serum albumin as standard.

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