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Protein Metabolism in Senescing Wheat Leaves[1]

DETERMINATION OF SYNTHESIS AND DEGRADATION RATES AND THEIR EFFECTS ON PROTEIN LOSS

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ABSTRACT

Wheat leaves (*Triticum aestivum* L.) at the moment of their maximum expansion were detached and put in darkness. Their protein, RNA and DNA contents, as well as their rates of protein synthesis and degradation, were measured at different times from 0 to 5 days after detachment. Rates of protein synthesis were measured by incorporation into proteins of large amounts of [^{3}H]leucine. Fractional rates of protein degradation were estimated either from the difference between the rates of synthesis and the net protein change or by the disappearance of radioactivity from proteins previously labeled with [^{3}H]leucine or [^{14}C]proline.

Protein loss reached a value of 20% during the first 48 hours of the process. RNA loss paralleled that of protein, whereas DNA content proved to be almost constant during the first 3 days and decreased dramatically thereafter.

Measurements of protein synthesis and degradation indicate that, in spite of a slowdown in rate of protein synthesis, an increased rate of protein breakdown is mainly responsible for the observed rapid protein loss.

Senescence is the series of changes which ordinarily precede death (21). It has been studied using intact plants, detached organs, and leaf discs in light and dark conditions (20, 22). It can be induced by detaching organs and/or imposing darkness. The most characterized changes in leaf senescence are losses of Chl, nucleic acids, and proteins (21, 26). The loss of proteins has been attributed to a proteolytic process. Experimental evidences supporting this assumption are an increase of free α-amino nitrogen and protease activity in the leaves (10, 12, 20, 24). However, *in vitro* measurements of proteolytic activity could not give accurate information on the role of proteolytic systems during the rapid loss of proteins in cells (14, 25).

RuBPCase[2] is the main component of soluble leaf proteins (50%), and it is lost during the initial stages of senescence (7, 16). It has been suggested that the loss of RuBPCase activity is due to a cessation of chloroplast protein and nucleic acid synthesis, coupled with a continued protein turnover (27). Darkness induces a marked loss in protein synthesis potential of chloroplasts isolated from *Nicotiana* leaves (23). Hence, it is not clear if the well documented loss of whole leaf proteins or RuBPCase during natural or induced senescence in leaves, is either due to a lower protein synthesis or to an activation of proteolysis, or to both processes (28).

In this report we describe quantitative measurements of protein synthesis and degradation in detached wheat leaves kept in the darkness. Our results experimentally support the earlier assumption that protein is mainly lost by an activated degradative process during senescence.

MATERIALS AND METHODS

Chemicals. L-Leucine and L-isoleucine were purchased from Sigma; L-proline and D-mannitol from Fluka; L-4,5[^{3}H]leucine (187 Ci/mmol) and L- U-[^{14}C]proline (285 Ci/mol) from Amersham. All other reagents were of the highest purity available.

Plant Material. Wheat (*Triticum aestivum* L. cv San Agustín, INTA) was generously supplied by the Balcarce Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. Plants were grown in a chamber at 25°C under 14 h photoperiod provided with irradiances of 525 w/m^2 supplied by Sylvania Gro-Lux fluorescent tubes; they were grown on vermiculite soaked in Hoagland nutrient solution. Thirteen days after sowing, the first leaves were fully expanded. At this moment they were detached and put in the dark at 25°C in tubes containing 0.5 ml of water. They were kept under these conditions until used for experiments. Water was changed every day.

Analytical Procedures. Leaves were weighed at the moment of detachment and at the moment of assay. For assays, they were homogenized individually unless otherwise stated, at 4°C in a glass homogenizer containing 3 ml of acetone. Acetone extracts containing Chl were separated by centrifugation at 2,000*g* during 15 min. This operation was performed twice. The resulting cleared pellets were processed for the following estimations: protein and nucleic acid content, and radioactivity in proteins in the case of labeling experiments. Nucleic acids were estimated by the procedure of Fleck and Munro (6) with the following modifications: pellets were resuspended in 0.3 N KOH and heated at 40°C during 1 h for alkaline hydrolysis of RNA. After that, suspensions were cooled and centrifuged for 15 min at 3,000*g* in order to eliminate cell walls. The resulting supernatants were processed as indicated in the original procedure. For the determination of protein content and radioactivity in proteins, pellets (which were obtained after acetone extraction from leaves) were treated with 2 ml of 0.25 N NaOH for 1 h at 50°C. Insoluble material was discarded by centrifugation. Aliquots of supernatant (0.5 ml) were assayed as indicated by Gornall *et al.* (9) for protein content. For radioactive content, 0.5 ml of supernatant were mixed with 50 μl of aqueous mouse liver homogenate containing 1 mg of protein used as carrier and reprecipitated with 1 ml of

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[2] Abbreviations: RuBPCase, ribulose 1,5-bisphosphate carboxylase.

20% (w/v) TCA; after 1 h at 4°C, samples were heated for 30 min at 90°C, cooled and centrifuged for 10 min at 3,600*g*, and washed successively with 2 ml each of ethanol/ether/chloroform (2:2:1, v/v), acetone and ethyl ether. Finally, pellets were dried for 15 min at 40°C, dissolved in 0.25 ml of HCOOH, and mixed with a scintillation solution containing 33% (v/v) Triton X-100 for radioactivity measurements. Leucine proportion in leaf protein was determined as previously indicated (4).

Rate of Protein Synthesis. At the indicated times after detachment, leaves were put under light (525 w/m^2). After 1 h they were transferred to tubes containing 10 to 100 mM-[^{3}H]leucine (300 μCi/mol) and the necessary amount of mannitol to maintain a constant concentration of solute equal to 100 mM. Labeling was started by submitting leaves to a strong air stream for 15 min in an illuminated hood. Radioactivity in proteins was determined 1, 3, and 5 h after precursor addition. The mass of [^{3}H] leucine incorporated per leaf and per day (V) was calculated from these data and from the specific radioactivity of labeling solution.

Effect of Large Amounts of Leucine on Incorporation of Trace Amounts of [^{14}C]Proline. Leaves were detached and put into 0.1 ml of a solution containing 50 μCi/ml of [^{14}C]proline (285 Ci/mol) and either mannitol, nonradioactive leucine, or isoleucine at a 100-mM concentration. Labeling was initiated with a strong air stream as indicated above and performed during 1 h. After that, total leaf protein radioactivity was determined.

Rates of Protein Degradation. Leaves were cut and put for labeling in tubes containing 0.3 μCi of [^{14}C]proline (285 Ci/mol) and 2.8 μCi of [^{3}H]leucine (187 Ci/mmol) in a total volume of 0.1 ml. During the next 0.5 h leaves were put under a strong air stream as indicated above and transferred to tubes containing 0.1 ml of a solution of nonradioactive proline and leucine (at a concentration of 100 mM each) and submitted again for 30 min, to the air stream. Five hours after detachment, leaves were put under darkness into tubes containing water. The total leaf protein radioactivity was assayed several times after detachment for 5 d.

RESULTS

Changes in Protein and Nucleic Acid Contents. Experiments were performed to compare changes in the content of protein, nucleic acids, and Chl of the first wheat leaf during natural and induced senescence. At different times after germination, the leaf was removed and analyzed for nucleic acid, protein, and Chl content. Results (not shown) indicated that all values increase during the first 10 d. At this moment, the first leaf was fully expanded and a steady state was attained in which no changes were observed in the parameters measured. At 15 d of culture, protein content, as well as Chl, started to decrease. From this point onward, the loss of protein, nucleic acids, and Chl was continuous until the leaf was dead (after 30 d). To obtain the values for senescence induced by detachment and darkness, fully expanded leaves (13 d old) were removed and placed in darkness as indicated in the "Materials and Methods". The same parameters were measured daily. The decrease in content of protein, nucleic acids, and Chl proceeded in a similar way to the decrease observed under natural conditions, except that the time required to attain the same values of loss of protein, nucleic acids, or Chl were significantly shorter.

From the above mentioned observations onward, experiments were perfromed only with detached leaves maintained in darkness (induced senescence) in order to obtain a rapid and reproducible response. Figure 1A shows that protein content per leaf was about 50% of the initial value 5 d after detachment. This decrease was accompanied by a dramatic decrease of RNA content, whereas DNA remained almost constant until 3 d after detachment. After that, DNA loss was significantly accelerated (Fig. 1B).

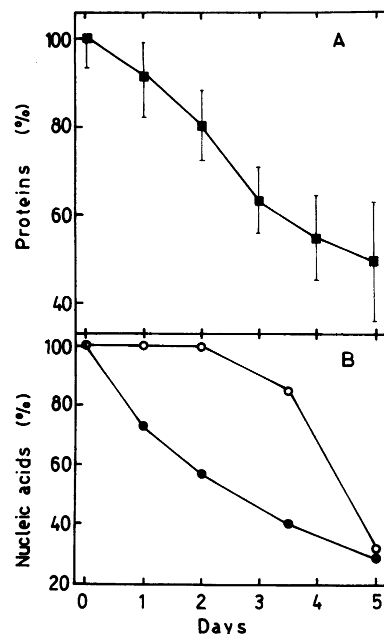

FIG. 1. Changes in protein and nucleic acid content. Changes in protein (A) and nucleic acids (B) of detached leaves in darkness. Data were expressed as percentages of their values in leaves at the moment of detachment; 100% values were 2.5 mg of protein per whole leaf, 0.079 mg of RNA per whole leaf, and 0.016 mg of DNA per whole leaf. Each point in (A) represents at least three leaves; SD is indicated by bars. Points in (B) represent pools containing six leaves. DNA (O); RNA (●). Average leaf weight at the moment of detachment was 76 mg.

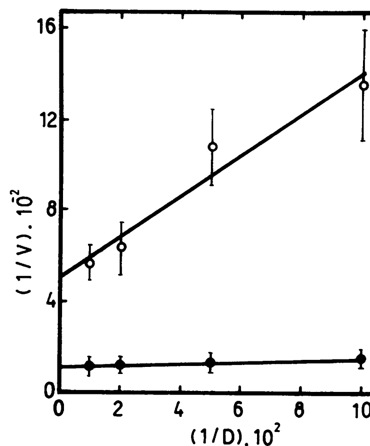

FIG. 2. Incorporation of large doses of radioactive leucine into leaf proteins. Leaves were labeled as indicated in "Materials and Methods," with doses of 10 to 100 mM [^{3}H]leucine during 1, 3, and 5 h. Rates of incorporation were estimated from curves in which three leaves per time of incorporation were analyzed. Reciprocal of the rates of incorporation ($1/V$; V = mg leu·leaf^{-1}·day^{-1}) are plotted *versus* reciprocal of the concentration of leucine used for labeling ($1/D$). (●), Leaves at the moment of detachment; (O), leaves 5 d after detachment. Bars, SD limits unless they were too small to be represented.

Protein Synthesis. To determine the concentrations of externally supplied [^{3}H]leucine necessary to saturate the endogenous precursor pools, the following studies were done: different concentrations of [^{3}H]leucine were supplied to leaves recently detached or maintained in darkness for different periods of time (up to 5 d) and incorporation into leaf proteins was measured.

Figure 2 shows the inverse of leucine incorporated into leaf protein against the inverse of the dose of leucine used for labeling

($1/V$ *versus* $1/D$). This study was conducted in leaves obtained either immediately after detachment or 5 d later at precursor concentrations from 10 to 100 mM. The rates of protein synthesis were calculated from the intercepts of the straight lines with ordinates, on the assumption that in this point ($1/D = 0$, $D = \infty$) the specific activity of the precursor in the leaf pool is that of the [^{3}H]leucine in the labeling solution. The values for velocity of incorporation ($Vmax$) at the intercepts were 8.2×10^{-3} mg of leucine per day at zero time and 1.95×10^{-3} mg of leucine per day for leaves 5 d after detachment, whereas their corresponding V values at $D = 100$ mM were $7.8 \pm 0.7 \times 10^{-3}$ mg of leucine per day and $1.69 \pm 0.21 \times 10^{-3}$ mg of leucine per day, respectively. These results indicated that precursor pool was 96% saturated for zero time and 86% saturated 5 d after detachment. Hence, it could be assumed that V values at $D = 100$ mM are not significantly different from those that would be obtained at $D = \infty$ when $V = V_{max}$.

The effect of 100 mM leucine on the incorporation of [^{14}C] proline into leaf proteins is shown in Table I. It indicates that the higher concentration of leucine used in Figure 2 for estimation of synthesis did not interfere with the incorporation of other amino acids (in this case proline). According to the experiments shown in Figure 2 and Table I, the fractional rates of synthesis were calculated from the milligrams of leucine incorporated per day to total leaf protein at 100 mM leucine (V), the leucine proportion in leaf (F_{leu}) and total leaf protein content (P) by the expression,

$$S = \frac{V}{F_{leu} \cdot P} \qquad (1)$$

Table I. *Effect of Large Amounts of Leucine on Incorporation of Trace Amounts of [^{14}C]Proline into Leaf Proteins*

Leaf proteins were labeled with [^{14}C]proline in trace amounts during 1 h in the presence of additions as indicated in "Material and Methods". Values are averages ± SD for three leaves.

Amino Acid Added	Concentration	[^{14}C]Proline Incorporation
	mM	dpm·mg fresh wt^{-1}
None		355 ± 36
L-Leucine	100	404 ± 27
L-Isoleucine	100	382 ± 40

Table II. *Estimation of Fractional Rates of Synthesis*

Fractional Rates of Synthesis (S) were estimated each day after detachment by equation 1 from the following data: mass of leucine incorporated per leaf and per day (V); leucine proportion in leaf protein (F_{leu}) and total leaf protein content (P).

Time after Detachment	V	F_{leu}	P	S
d	*μg leu·d^{-1}*	%	*mg*	*%·d^{-1}*
0	7.2 ± 0.7	10.25 ± 0.5	2.48 ± 0.15	2.85 ± 0.28
1	7.1 ± 2.0	10.40 ± 1.2	2.28 ± 0.17	3.00 ± 0.90
2	6.9 ± 1.0	12.10 ± 2.1	1.98 ± 0.15	2.88 ± 0.55
3	5.1 ± 1.0	10.35 ± 0.6	1.57 ± 0.10	3.12 ± 0.53
4	3.2 ± 0.5	10.55 ± 0.7	1.40 ± 0.14	2.18 ± 0.22
5	1.5 ± 0.2	12.60 ± 2.9	1.32 ± 0.14	0.90 ± 0.14

Table III. *Estimation of Fractional Rates of Protein Breakdown*

Values of synthesis obtained in Table II at the initiation and at the end of each day were averaged. The net protein changes were calculated from Figure 1 as follows: the variation of protein over a 1-d period was divided by the value of protein content in the middle of that period. Fractional rates of protein breakdown were obtained from the difference beween synthesis and net protein change.

Time Interval after Detachment	Average Protein Synthesis	Protein Change	Protein Breakdown
d		*%·d^{-1}*	
0–1	2.9	−9.5	12.4
1–2	2.9	−12.9	15.8
2–3	3.0	−23.8	26.8
3–4	2.7	−13.6	16.3
4–5	1.5	−9.5	11.0

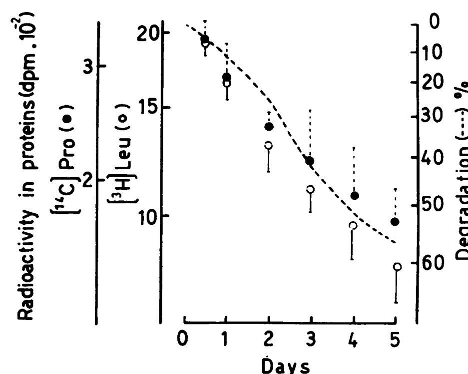

FIG. 3. Disappearance of protein radioactivity from leaves. Each leaf was labeled with [^{3}H]leucine (O) and [^{14}C]proline (●) immediately after its detachment as indicated in "Materials and Methods." Total protein radioactivity was determined in groups of three leaves several times after detachment. Bars, SD limits. (---), decay curve calculated from the fractional rates of degradation obtained in Table III from the difference between synthesis and net protein change.

Table II indicates estimations of S obtained from V values and their corresponding values for F_{leu} and P for leaves at several times after detachment. These data showed that synthesis was not modified significantly until the 4th d after detachment. This experiment also shows that V values for 0 and 5 d after detachment were close to those obtained in the experiment of Figure 2.

Estimation of Fractional Rates of Protein Breakdown. Protein degradation was estimated from the data of protein synthesis obtained each day (Table II) and from the values of net protein change (Fig. 1). Since no variation in protein mass was observed in 10- to 15-d old leaves, it was assumed that protein breakdown at the moment of detachment (13th d) should be equal to the initial rate of synthesis (3%). Hence, results in Table III, taken on a daily basis, indicated a 4-fold increase of breakdown rate during the 1st d. Since synthesis did not vary significantly, breakdown should be mainly responsible for the protein loss observed.

Disappearance of Protein Radioactivity from Leaves. To confirm the quantitative estimation of protein degradation obtained indirectly, experiments were done to measure the disappearance of radioactive proteins from leaves. [^{14}C]Proline and [^{3}H]leucine in trace amounts were supplied to freshly cut leaves. A chase of radioactivity was done by putting each one of the leaves in 100 mM of each nonlabeled amino acid. Radioactivity in proteins was measured during the following 5 d. Figure 3 indicates that disappearance of label from leaf proteins labeled either with [^{3}H] leucine or [^{14}C]proline was almost the same. In addition, these decay curves were coincident with that calculated from the fractional rates of degradation obtained by the indirect method in Table III.

DISCUSSION

The observed decrease in leaf protein is in agreement with previous reports based on studies on detached wheat leaves (2)

under continuous light or darkness (24), and other plant materials, such as oat leaves (13), barley leaves (16), bean pod tissue (17), and cotyledons of mustard plant (15). In a wider sense, protein, RNA, and DNA changes (Fig. 1) are in agreement with those observed in several kinds of plant (21, 26). The loss of proteins is certainly a dominant feature of leaf senescence. As it has been stated above, these changes can be attributed to variations in the rates of synthesis, degradation, or to both. The incorporation of trace amounts of radioactive aminoacids into proteins (19) indicate that protein synthesis is still going on. However, this method cannot give accurate results on the magnitude of the process.

The approach used for the measurement of rates of protein synthesis with saturating doses of amino acid precursor (Fig. 2; Table II) has not been used in plants. The exception is the work of Brady and Tung (2) on detached wheat leaves which used increasing concentrations of radioactive valine until constant values of incorporation were obtained. The advantages of the use of this method in animal cells have been discussed in detail by Scornik *et al.* (18). From Figure 2 it is possible to conclude that saturation of precursor pool is more easily attainable in fresh leaves than in leaves after 5 d of detachment. This could either be due to the increase in the level of free amino acids that occurs in senescent leaves (13), or to a lesser ability of leaves for the uptake of amino acids after 5 d detachment. Kemp and Sutton (11) obtained differential uptake rates of tritiated leucine in tobacco callus of different ages. On the other hand, the chosen concentration of 100 mM leucine seems to be adequate to obviate pool differences since it saturates them up to 91% in the average. This concentration also seems not to exert any influence on the incorporation of other amino acids.

The fractional rates of protein synthesis shown in Table II, and the time course of protein change shown in Figure 1 provide an insight into the balance between synthesis and degradation. Since synthesis is about 3% per day, one would conclude that 9.5% protein loss observed during the first day is mainly due to an increased degradation. However, it is necessary to quantitate both synthesis and breakdown, at least on a daily basis, in order to have a better description of the phenomenon. This was calculated in Table II, which shows more clearly the importance of breakdown during protein loss. A complete cessation of synthesis would account for 13% loss of protein after 5 d, which is far lower than the observed value. The estimation of rates of protein breakdown made from measurements of synthesis, and the protein mass change have been successfully used for liver (18), muscle (8), and kidney (3). As shown in Table III, it has the disadvantage that it could be influenced by errors arising from measurements of either synthesis or protein change. However, in this study it proved to be reliable when based on results obtained by the conventional procedure. The experiment in Figure 3, using proline and leucine as precursors, was designed to avoid the major difficulty in this kind of study, which consists in the recycling of label for synthesizing new protein. Results obtained using this direct method are in accordance with those obtained by the indirect one, which is not influenced by the reuse of label. Hence, the contribution of protein breakdown to the loss of protein from detached leaves under darkness has been determined by two procedures. Furthermore, this is the first work in which the rate of protein breakdown during the onset of leaf senescence has been accurately estimated.

Increase of *in vitro* proteolytic activity during leaf senescence (1, 5, 16, 24) has been detected about 2 d after detachment (24), at which time the leaf has already lost a considerable amount of protein. Moreover, Peterson and Huffaker (16) showed that in detached primary barley leaves, the rapid decline of RuBPCase protein is negatively correlated with the increase in proteolytic activity (measured with azocasein as substrate). In addition, results on yeast (25) have shown that caution must be observed when trying to directly correlate results from the *in vitro* activity of proteases with *in vivo* proteolytic processes.

These studies suggest that increases of proteolytic activity in leaf extracts may have no relevance for protein loss in the whole leaf. Therefore, further work is necessary to evaluate the mechanism underlying the enhanced degradation of protein associated with the onset of leaf senescence.

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