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Autodigestion in Crude Extracts of Soybean Leaves and Isolated Chloroplasts as a Measure of Proteolytic Activity[1]

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ABSTRACT

Two methods of measuring protein breakdown resulting from self-digestion during incubation in extracts of soybean leaves were examined. The release of free α-amino-nitrogen was measured with ninhydrin, and the disappearance of the large subunit of ribulose bisphosphate carboxylase (RuBPcase) was followed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Rates of protein breakdown were measured as a function of temperature, pH, and leaf developmental stage and in the presence of various proteinase inhibitors. These treatments had differential effects on apparent proteolysis, depending on the method used. Determination of the ratio of α-amino-nitrogen plus peptide bond-nitrogen to α-amino-nitrogen indicated that the ninhydrin method detected the activity of exopeptidases preferentially. The disappearance of the large subunit of RuBPCase as shown on gels was due primarily to the activity of endopeptidases. The sensitivity of the two types of proteolytic degradation to proteinase inhibitors differed.

Determination of temporal changes in proteolytic activity during leaf development showed that total proteolytic activity, measured by either method, increased during leaf expansion and maturation and decreased during senescence. Incubation of intact isolated chloroplasts at 37 C resulted in the breakdown of the large subunit of RuBPcase, although the chloroplasts contained no measurable proteinase activity as determined by the release of α-amino-nitrogen during the incubation. No acid proteinase (pH 4.5) activity was detected in the chloroplasts when hemoglobin was used as a substrate. These results indicate that the proteinases which break down RuBPCase in isolated chloroplasts may not be detectable by conventional assay procedures.

Leaf senescence in many plants, and especially in monocarpic crop plants, is accompanied by breakdown of leaf protein and reutilization of the hydrolysis products by the developing seeds (9–11, 21, 26). It has been suggested that the amount of protein which accumulates in the seeds is related to the extent of leaf protein breakdown (11, 19). Attempts have been made to correlate leaf protein breakdown with the levels of various proteinases in the leaves and, in some cases, a correlation was found (9, 11, 26, 37), whereas, in others, there appeared to be no relation between proteinase levels and protein breakdown (1, 28, 32).

One of the major problems with such experiments is that there is no ideal way to measure proteinase activity. Many investigators have relied on synthetic proteinase substrates or animal proteins (collagen, hemoglobin, casein) as substrates. Recently, a few studies have been carried out in which RuBPCase[4] was used as a substrate (23, 25, 35, 36). RuBPCase is the most abundant soluble protein in the leaf, accounting for up to 50% of the soluble protein (16, 17, 37). Hydrolysis of RuBPCase or any other protein substrate can be measured in several ways: by the release of ninhydrin-reactive material (10–12, 23), by the loss of immunochemical determinants (35–37), or by the breakdown of polypeptides as ascertained by SDS-acrylamide gel electrophoresis (33).

As part of our study on the role of proteinases in protein breakdown during leaf senescence, we have examined the hydrolysis of the LS of RuBPCase during the autodigestion of crude leaf extracts. Proteolysis was measured by the release of ninhydrin-reactive material and the disappearance of the LS of RuBPCase on SDS-polyacrylamide gels. Our results show that the two methods probably measure different complements of proteinases. The discrepancy between the two methods was most obvious in isolated chloroplasts, where we found no apparent proteinase activity as measured with ninhydrin, whereas endopeptidic cleavage of the LS of RuBPCase as measured on gels was quite rapid.

MATERIALS AND METHODS

Plant Material. Soybean [*Glycine max* (L.) Merr. cv. Steele] plants were grown in pots in the greenhouse in La Jolla, CA, without supplemental lighting. The seeds were treated with a *Rhizobium* inoculum and planted in a mixture of sand, peatmoss, horticultural Perlite, and slow-release fertilizer. Leaf samples were collected as needed, usually from the fourth, fifth, or sixth trifoliate node, and transported to the laboratory in ice. Plants whose leaves were used for isolating chloroplasts were put on a cycle of 18 h dark/6 h light for several days and sampled at the end of a dark period.

Extraction. Leaves were weighed, cut into small pieces, and homogenized in 4 volumes 50 mM K-phosphate buffer (pH 7.5) containing 1% insoluble PVP (Sigma) and 20 mM Na-metabisulfite. Homogenization was for 90 to 120 s at 3 C at top speed with a Polytron homogenizer. Homogenates were filtered through four layers of cheesecloth and centrifuged for 10 min at 12,000*g*. Supernatants were dialyzed for 2 to 5 h at 5 C against 100 volumes 25 mM K-phosphate (pH 7.5).

Determination of Protein and Chl. Protein was measured by the method of Lowry *et al.* (20) using BSA as a standard. Aliquots of leaf extract were precipitated with trichloroacetic acid at a final concentration of 7.5% (w/w) and the precipitate redissolved in 100 mM NaOH after centrifugation and removal of the supernatant. No correction was made for phenolics because it was assumed that

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[4] Abbreviations: RuBPCase, ribulose bisphosphate carboxylase; LS, large subunit of RuBPCase; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide.

the homogenization with polyvinylpyrrolidone and the precipitation with trichloroacetic acid removed most phenolics from the protein sample. Chl was estimated by its A at 665 nm. An aliquot of 0.1 ml of the leaf extract was mixed with 0.9 ml H_2O and 4.0 ml acetone, and the mixture was left in the dark at 3 C for 30 min before the A was read.

Autodigestion and Proteolytic Activity. Dialyzed extracts (usually 200 μl) were incubated at 37 or 45 C for 30 to 120 min with shaking in a water bath. In most experiments, 50 μl Na-acetate buffer was added to a final concentration of 200 mM and a pH of 4.5 or 5.0. The reaction was terminated by adding 250 μl 10% (w/w) trichloroacetic acid. The precipitated proteins were removed by centrifugation after standing at 3 C for 30 min. Free amino groups in amino acids and peptides in the supernatant were quantitated with ninhydrin using leucine as a standard as described by Spies (30). Less than 200 μl trichloroacetic acid supernatant was used for ninhydrin assays, and no correction for trichloroacetic acid was made. This measure of proteolytic activity is referred to as the release of ninhydrin-positive material and is expressed as μmol released/h incubation·mg protein.

A second measure of proteolytic activity was obtained by measuring the relative intensity of staining of the LS of RuBPCase on a polyacrylamide gel. The protein precipitated by trichloroacetic acid after autodigestion was extracted with 80% acetone to remove excess acid and dissolved in denaturation buffer (3) by heating for 4 min to 100 C, and an aliquot was applied to the well of a slab gel. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis using the method of Laemmli (18) modified slightly (3). Gels were stained with Coomassie brilliant blue, destained, and scanned with a Joyce-Loebl microdensitometer. The area under the peak corresponding to the LS of RuBPCase was determined by weighing the paper and converting weight to area. We established that there was a linear relationship between the area under the peak and the amount of protein on the gel by running gels with different amounts of protein; values outside the linear range were rejected.

Chloroplast Isolation. Leaves were cut into small pieces with scissors and then chopped with a mechanical razorblade chopper for 2 min in 2 volumes isolation medium consisting of 10 mM KCl, 1 mM $MgCl_2$, 1% BSA, 1 mM EDTA, 1% PVP-40 (Sigma), 20 mM sodium metabisulfite, and 12% (w/w) sucrose in 50 mM Tricine buffer (pH 7.5). The filtrate was loaded on a gradient containing, in sequence from the bottom of the tube, 4 ml 60% sucrose, 8 ml of a linear gradient from 60 to 43% sucrose, 5 ml 43% sucrose, 10 ml of a linear gradient from 43 to 30% sucrose, and a final 2 ml 30% sucrose; centrifugation was for 5 min at 3000g and then for 10 min at 13,000g, essentially as outlined by Miflin and Beevers (22). Sucrose solutions were made up w/w in 50 mM Tricine-NaOH buffer (pH 7.5) containing 1 mM EDTA, 1 mM $MgCl_2$, 10 mM KCl, 2 mM mercaptoethanol, and 0.1% BSA. The gradients were fractionated starting at the top with a Büchler Auto Densiflow IIC gradient apparatus. Gradient fractions (1 ml) were analyzed for Chl, protein, and NADPH trioseP dehydrogenase activity as well as for the autodigestive proteolytic activity (ninhydrin) and hemoglobin-digesting activity at pH 4.5. Incubations for autodigestive and hemoglobin-digesting activity were done at 45 C for up to 3 h if activities were low. Autodigestive proteolytic activity visualized on gels was done on selected samples at the top of the gradient (soluble proteins) and pooled chloroplasts present between 47 and 52% sucrose.

Enzyme Assays. NADPH trioseP dehydrogenase was measured as described by Gibbs and Turner (14). Hemoglobin-digesting activity was measured using a modification of the Frith *et al.* (13) procedure. Extract (100 μl) was incubated for 90 min at 45 C with 250 μl hemoglobin (6 mg/ml, Sigma Bovine Type I) made up in 200 mM Na-acetate buffer (pH 4.5). After incubation, the reaction was stopped by adding trichloroacetic acid to a final concentration of 5% (w/w). Release of trichloroacetic acid-soluble α-amino-N was measured with ninhydrin (31).

RESULTS

Autodigestion in crude extracts exhibited different pH profiles, depending on the method used to measure proteolysis. The release of ninhydrin-positive material occurred over a wide pH range without a definite maximum; activities at pH 5, 7 and 9 were roughly equivalent (Fig. 1). SDS-polyacrylamide gel electrophoresis of the autodigestion products indicated that the hydrolysis of the LS of RuBPCase occurred in a much narrower pH range with a maximum around pH 4 to 5 (Fig. 2). There was much less breakdown of the LS of RuBPCase or of the other polypeptides visible on the gel between pH 6.5 and 9.2 than at pH 4 or 5, indicating that the endopeptidases in leaf extracts were most active in the acidic pH range. Longer incubations (4 h) resulted in almost total disappearance of the LS or RuBPCase in the pH 3 to 5 range (data not shown). Some degradation of the LS or RuBPCase at pH 10.0 may also occur (Fig. 2), but this was not investigated

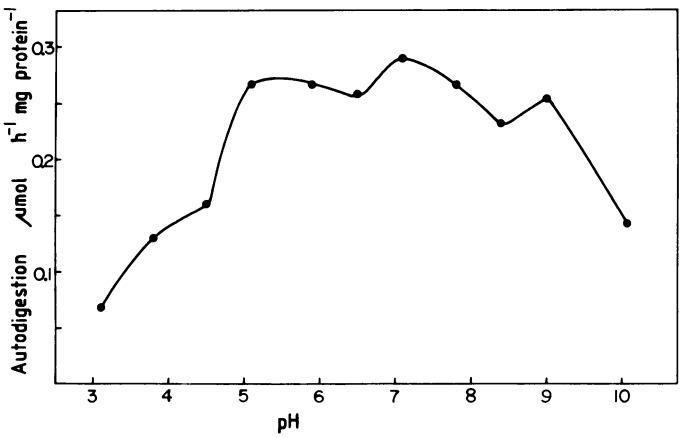

FIG. 1. pH profile of proteolytic degradation of proteins in extracts of soybean leaves: amino acid release. Extracts were dialyzed for 3 h against 50 mM Na-acetate (pH 5.0), 50 mM K-phosphate (pH 7.5), or 50 mM Tris-HCl (pH 9.0) and adjusted to pH values indicated. Acetate was used for pH 3.1 to 5.1, phosphate for 5.9 to 7.8, and Tris for 8.4 to 10.0. Incubation for 2 h at 37 C was followed by trichloroacetic acid precipitation and determination of ninhydrin-positive material in the supernatant.

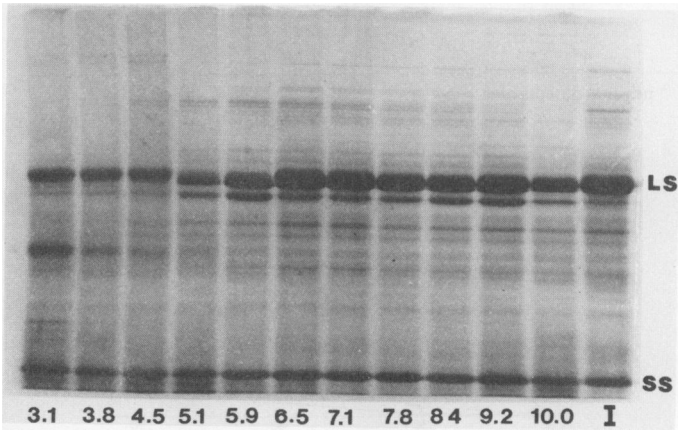

FIG. 2. pH profile of proteolytic degradation of protein in extracts of soybean leaves: endopeptidic cleavage. Extracts were dialyzed and adjusted as in Fig. 1. After incubation for 2 h at 37 C, the polypeptides were separated by SDS-polyacrylamide gel electrophoresis. Numbers indicated pH values; I, initial; SS, small subunit of RuBPCase.

further.

The relative contribution of exo- and endopeptidases to the release of ninhydrin-positive material during autodigestion was determined by measuring the ratio of α-amino-N + peptide-N over α-amino-N in trichloroacetic acid supernatants of autodigested leaf extracts. The trichloroacetic acid supernatants were extracted with ether to remove the trichloroacetic acid; half the sample then was hydrolyzed with 6 N HCl (110 C for 18 h) and ninhydrin-positive material was determined before and after the hydrolysis. The results were expressed as a ratio of free α-amino groups after acid hydrolysis to free α-amino groups before hydrolysis. When autodigestion was performed at pH 5.0 and 7.0, we obtained ratios of 3.4 and 2.2, respectively. These results indicate that measurements of ninhydrin-positive material reflected primarily the action of exopeptidases. However, the contribution of endopeptidases to the release of ninhydrin-positive material was greater at pH 5.0 than at pH 7.0.

Breakdown of protein and release of ninhydrin-positive material during autodigestion was dependent on the temperature of incubation and increased from 23 C to 45 C. Release of ninhydrin-positive material was linear over the 90-min incubation period and doubled between 30 C and 45 C (Table I). There was, on the other hand, a 4-fold increase in the loss of LS detected on gels between those same temperatures (Fig. 3). These data indicate that the endopeptidases were relatively more active than the exopeptidases at the higher temperatures. We could not detect any loss of LS during incubation of leaf extracts at 23 C, although release of ninhydrin-positive material was easily detectable at this temperature.

The sensitivity of both types of proteolytic degradation to inhibitors of proteinase activity was tested and found to differ. PMSF at 2 mM, and *p*-chloromercuribenzoate at 0.2 mM inhibited the release of ninhydrin-positive material by 38% (Table II) without affecting the loss of LS indicated on gels after electrophoresis of the digestion products (Fig. 4). NEM at 2.5 mM inhibited the release of ninhydrin-positive material by 30% (Table II) and also inhibited the loss of LS on gels (Fig. 4). EDTA at 3 mM had little effect on proteolysis measured by either method. These results indicate that the release of ninhydrin-positive material at pH 5.0

Table I. *Effect of Incubation Temperature on Proteolytic Degradation of Proteins in Soybean Leaf Extracts*

Dialyzed extracts were made 0.2 M with respect to Na-acetate buffer (adjusted to pH 4.5) and incubated at different temperatures. Ninhydrin-positive material was measured in the trichloroacetic acid supernatant, and an aliquot of the trichloroacetic acid precipitate was subjected to SDS-polyacrylamide gel electrophoresis to determine the amount of LS of RuBPCase. All numbers reflect rates calculated on the basis of several time points (30, 60, 90, 120 min).

Temperature	Proteolytic Activity	
	Release of α-Amino Groups	Loss of LS of RuBPCase
C	*nmol amino acid/mg protein·h*	*% loss/h*
23	77	nd[a]
30	237	10
37	314	22
45	481	50

[a] nd, not detectable.

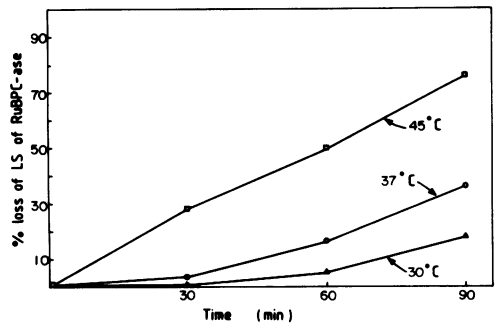

FIG. 3. Effect of incubation temperature on proteolytic degradation of proteins in soybean leaf extracts. Conditions as in Table I. Per cent loss of LS of RuBPCase was calculated by quantitating the bands of LS on the gels after staining with Coomassie blue.

Table II. *Effect of Proteinase Inhibitors on Proteolytic Degradation of Proteins in Soybean Leaf Extracts*

Dialyzed extracts (at pH 7.5) were preincubated for 90 min at 3 C with various proteinase inhibitors; the pH was lowered to pH 4.5 by the addition of 0.2 vol 1 M Na-acetate (pH 4.5). Extracts then were incubated at 37 C for different periods of time, and ninhydrin-positive material in the trichloroacetic acid supernatant was determined. Numbers reflect rates calculated on the basis of several time points during a 3-h incubation.

Proteinase Inhibitor	Concentration of Inhibitor	Release of α-Amino Groups	
		Amount	Inhibition
	mM	*nmol/mg·h*	*%*
Control		55	
EDTA	3	50	10
PMSF	2	34	38
p-Chloromercuribenzoate	0.2	34	38
NEM	2.5	38	30
NEM	5.0	30	45

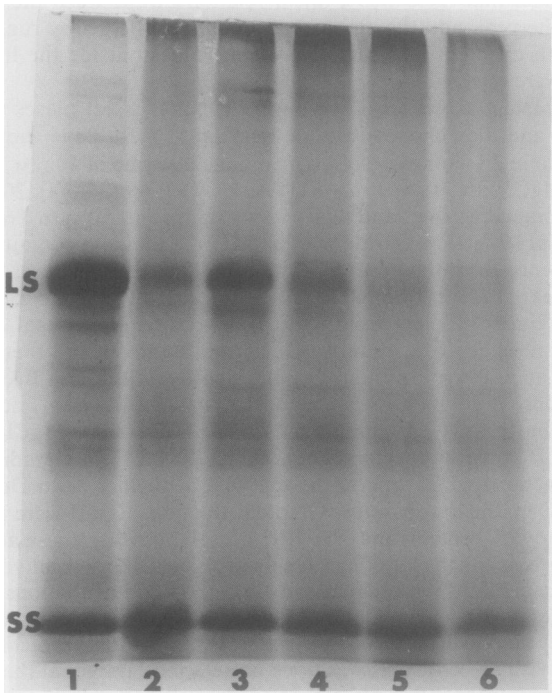

FIG. 4. Effect of proteinase inhibitors on proteolytic degradation of proteins in soybean leaf extracts. Conditions were as in Table II. Lanes show protein remaining after incubation for 3 h: on ice (lane 1), at 37 C (lane 2), or at 37 C with: (lane 3), 2.5 mM NEM; (lane 4), 0.2 mM *p*-chloromercuribenzoate; (lane 5), 3 mM EDTA; or (lane 6), 2 mM PMSF. After the incubation, the polypeptides were separated by SDS-polyacrylamide gel electrophoresis. SS, small subunit. Only NEM visibly retarded the hydrolysis of the LS.

is caused in part by an exopeptidase sensitive to PMSF—possibly carboxypeptidase, an enzyme known to be inhibited by PMSF (27, 30, 34)—and in part by an endopeptidase inhibited by NEM.

To study the temporal changes in autodigestive activity during leaf ontogeny, we used leaves at the fifth node of greenhouse-grown plants. Leaf maturation was accompanied by an increase in the amount of protein/leaf during the first 2 weeks of growth, followed by a decline during the next 3 weeks (Fig. 5). Autodigestive activity as measured by the release of ninhydrin-positive material followed approximately the same pattern but remained high when the leaves were losing protein. The activity dropped sharply when the leaves were yellowing (11 weeks), possibly as a result of the disappearance of substrate. The different patterns of change of protein and autodigestive activity during leaf ontogeny are reflected in the specific activity of the proteolytic activity which was highest when the leaf protein levels were rapidly declining (Fig. 5). The loss of LS of RuBPCase during autodigestion was determined with SDS gels at leaf maturity (8 weeks) and early senescence (10 weeks). The loss of the LS was found to be 13 and 45%/h in extracts of mature and sensescent leaves, respectively, when the extracts were incubated at pH 5.0 at 37 C. It seems that the two methods do not measure enzymes with the same developmental patterns, as there was no similar increase in autodigestive activity indicated by the release of α-amino-N.

Maturation and senescence of leaves have been shown to be accompanied by dramatic changes in chloroplast ultrastructure (5, 15, 37) and by losses of RuBPCase activity and protein (23, 35, 37). These observations prompted us to find out if autodigestive activity is associated with isolated intact chloroplasts. The organelles present in homogenates of leaves were fractionated on a sucrose gradient. The distribution of Chl in such a gradient shows a major band at 38% sucrose and a minor band at 50% (Fig. 6). The distribution of NADP-dependent trioseP dehydrogenase in the same gradient shows that most of the activity is present in the load portion of the gradient and that there is a small peak at 50% sucrose (Fig. 6). No enzyme activity was associated with the large peak of Chl at 38% sucrose. Examination of the organelles present at 38 and 50% sucrose with a light microscope showed that only those present at 50% were highly refractile. Together, these data support the interpretation that intact chloroplasts formed a band at 50% sucrose, whereas the main peak of Chl at 38% represents broken chloroplasts.

Proteolytic activity on the gradient was determined in three different ways. We assayed for the digestion of hemoglobin and

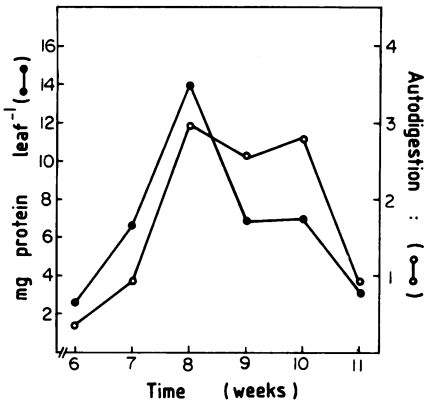

FIG. 5. Temporal changes in protein and proteolytic degradation of proteins during incubation of extracts of soybean leaves during leaf development and senescence. Dialyzed extracts were made 200 mM with respect to Na-acetate buffer (pH 4.5) and were incubated for 2 h at 45 C. Ninhydrin-positive material in the trichloroacetic acid supernatant was assayed. All values are expressed as mol α-amino N released per h incubation per leaf.

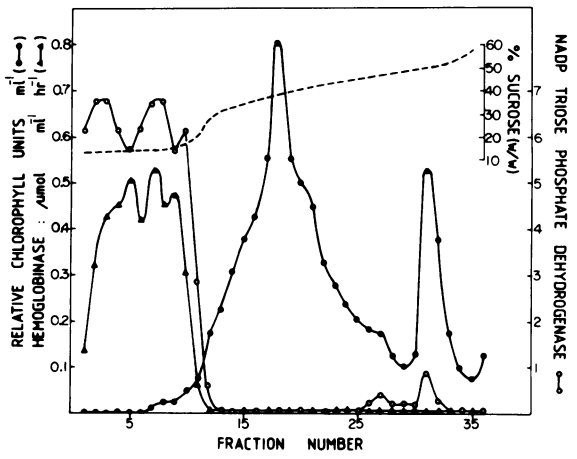

FIG. 6. Distribution of Chl, NADPH trioseP dehydrogenase activity, and hemoglobin-digesting activity on isopycnic sucrose gradient of soybean leaf homogenate. Ten ml chopped leaf filtrate was loaded on the gradient which was centrifuged at 3,000*g* for 5 min and then at 13,000*g* for 10 min; 1-ml fractions were collected and assayed as described.

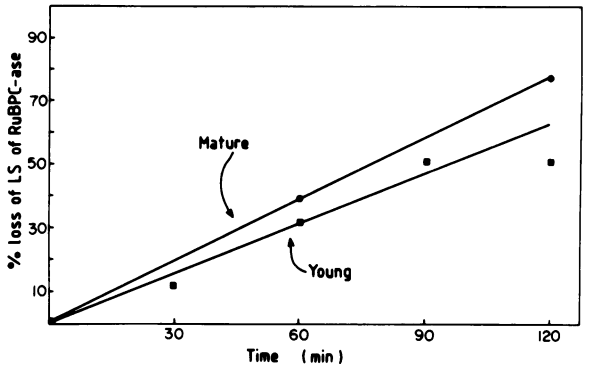

FIG. 7. Proteolytic degradation of LS of RuBPCase during incubation of intact chloroplasts isolated on sucrose gradients from young and mature leaves. Chloroplasts were isolated on sucrose gradients (Fig. 6) and the pH was adjusted to 4.5 by making the pooled fractions 200 mM with respect to Na-acetate buffer (pH 4.5). Incubations at 45 C was carried out up to 2 h. Chloroplasts and released proteins were precipitated with trichloroacetic acid (7.5% final concentration) and the polypeptides were separated by SDS-polyacrylamide gel electrophoresis. LS of RuBPCase was quantitated by scanning the stained gels and measuring the area under the peak corresponding to the LS.

the release of ninhydrin-positive material at pH 4.0, the pH optimum for this reaction (29). Hemoglobin-digesting activity was present in the load portion of the gradient, but no activity was associated with either the broken or the intact chloroplasts (Fig. 6). The release of ninhydrin-positive material during autodigestion at pH 5.0 followed exactly the same pattern, but the absolute values for the amount of α-amino-N released/h incubation were lower by a factor of 3.4 (data not shown). Larger (3-fold) aliquots of intact chloroplasts were incubated for longer (2-fold) periods of time, as compared to the load portion of the gradient, to make sure that there was no minor peak of activity associated with the intact chloroplasts. The degradation of LS of RuBPCase was followed by SDS-polyacrylamide gel electrophoresis of the digestion products after incubation at 45 C (Fig. 7). The rate of loss of LS during the incubation of intact chloroplasts at 45 C was 39%/h; a similar rate of loss was observed when the load portion of the gradient was autodigested (results not shown). These experiments were repeated with young, expanding leaves and similar results were obtained. The intact chloroplasts contained no hemoglobin-

digesting activity and released no detectable ninhydrin-positive material upon incubation, but the rate of loss of LS was 30%/h. This rate was again similar to that obtained when incubating aliquots of the load portion of the gradient. Attempts to repeat the experiments with chloroplasts from senescing leaves proved unsuccessful as we were unable to obtain intact chloroplasts from such leaves. Incubation of the intact chloroplasts with proteolytic inhibitors showed that PMSF or EDTA did not affect the breakdown of LS, whereas NEM inhibited it by 24%.

DISCUSSION

The purpose of this study was to examine and compare methods which measure proteolysis in crude leaf extracts by allowing such extracts to autodigest. Two methods were used to assess protein breakdown: the release of ninhydrin-positive material, and the loss of LS of RuBPCase, the most abundant large polypeptide in the leaf extracts. Although these two activities were correlated in some situations, it is clear that the release of ninhydrin-positive material is not necessarily a reliable measure of endopeptidic protein degradation. For example, although the release of ninhydrin-positive material is high above pH 7.0, on SDS gels there appears to be little endopeptidic cleavage of the proteins in that pH range.

The rapid breakdown of LS of RuBPCase upon incubation indicates that young and mature leaves have sufficient proteolytic activity to break down RuBPCase in a relatively short time at ambient temperatures (30 C). Such observations indicate that the proteinases and the proteins do not occur in the same compartments and that the control of proteolysis may depend on changes in compartmentation, rather than on changes in proteinase levels. Acid proteinases have been shown to occur in the central vacuole (2, 4), whereas a considerable portion of a leaf's soluble protein is in the chloroplast. Breakdown of chloroplast proteins during late senescence may occur when chloroplasts enter the vacuole in an autophagic process (36) or when vacuolar proteinases enter the chloroplast. Since chloroplasts often lie closely apposed to the tonoplast, it is possible that proteinase may be taken directly into the chloroplasts without first passing into the cytosol.

Our results with intact chloroplasts raise some interesting questions about the presence of proteinases within the chloroplasts. When isolated chloroplasts were cultured in darkness for several days, their protein content declined by 20% (6, 7), indicating that they contained proteolytic enzymes. It has also been shown that the processing of the precursor of the small subunit of RuBPCase involves a proteolytic cleavage carried out by an intrachloroplastic proteinase (8). Yet, most attempts to show that isolated chloroplasts contain proteinases which can be measured with exogenous substrates have failed, and there is only one short report indicating that isolated chloroplasts contain significant levels of proteinase activity (24). With the exception of the reports on processing of the small subunit of RuBPCase, we are not aware of any reports showing that isolated chloroplasts contain proteinases which can be detected by using the endogenous chloroplast proteins.

Our experiments confirm that chloroplasts are devoid of apparent proteolytic activity, as indicated by the release of ninhydrin-positive material from endogenous substrates and/or hemoglobin. Since the chloroplasts were incubated at 37 C in a medium without sucrose or other osmoticum, it seems unlikely that they remained intact and that the lack of apparent hemoglobin-digesting activity was due to the inability of the hemoglobin to get into the chloroplasts. It seems more likely that the exopeptidase activities are so low in the chloroplasts that the proteolytic digestion which does occur does not result in the release of detectable levels of free amino acids in the trichloroacetic acid supernatant. In addition, the chloroplast proteinases may have a greater affinity for the endogenous substrates than for exogenous substrates such as hemoglobin. Our results showing the breakdown of RuBPCase when isolated chloroplasts are incubated do not allow us to conclude that this degradation is caused by intrachloroplastic proteinases, rather than cytoplasmic or vacuolar enzymes adhering to the outside of the chloroplasts as a result of chloroplast isolation.

The data presented here emphasize the importance the method for measuring proteolytic activity has on interpreting the results. Methods based on the reaction of free α-amino groups with ninhydrin may not be sensitive enough if the organelles or partially purified enzyme preparations are devoid of exopeptidase activity. The difficult question about the existence and nature of chloroplastic proteinases should probably be investigated using methods suitable for the detection of low levels of endopeptidase activity.

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