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Changes in the endopeptidase pattern of wheat leaves during senescence

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Abstract

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Peptide hydrolase activities present in young and senescing flag leaves of wheat (*Triticum aestivum* L.) were fractionated on DEAE-cellulose columns. Part of the endopeptidase activity was not bound to DEAE-cellulose at pH 7.5 (peak I) and had a broad pH optimum (against acetylated casein) between 6 and 9. The other peaks (II A and II B) eluted in the NaCl gradient showed a pH optimum between 4 and 7. During senescence the endopeptidase pattern changed markedly in favor of peak I. Glutamine synthetase and nitrate reductase in extracts from young leaves were rapidly inactivated after addition of peak I-eluate, while peak II-eluate was considerably less effective.

Introduction

The proteolytic activity in crude extracts from leaves of cereals and grasses increases during senescence and the pH optimum changes from the slightly acidic to the neutral range (Martin and Thimann 1972, Feller et al. 1977, Thomas 1978). The observed changes in the pH optima for total proteolytic activity suggest different enzyme patterns in non-senescing and senescing leaves. Several endopeptidases were separated from leaf extracts of wheat (Frith et al. 1978), barley (Ragster and Chrispeels 1981) and oat (Drivdahl and Thimann 1977).

Glutamine synthetase (Batt and Wallace 1983) and nitrate reductase (Wallace and Shannon 1981, Wray and Kirk 1981, Batt and Wallace 1983) are very susceptible to inactivation by proteolysis. In young wheat leaves, the inactivation of glutamine synthetase and nitrate reductase was accelerated by the addition of extracts from senescing leaves (Streit and Feller 1982). Enzyme inactivation by proteolysis may depend on the activity and the quality of peptide hydrolases (Holzer and Heinrich 1980). The inactivation of glutamine synthetase and nitrate reductase by the addition of extract from senescing leaves could be due to the increase of total proteolytic activity but also to a change in the endopeptidase pattern during senescence.

The aim of this work was to analyse the changes in the endopeptidase pattern during senescence and to investigate glutamine synthetase and nitrate reductase inactivation by the different peaks.

Materials and methods

Plant materials

Flag leaves of winter wheat (*Triticum aestivum* L., cv. 'Probus') were collected from a field in Zollikofen near Bern at various stages of development during summer 1981. The leaves were transported in plastic bags on ice to the laboratory and stored frozen (–18 °C). Young leaves were collected on May 21 and used as source for proteolytic activity and glutamine synthetase activity. Flag leaves collected on July 30 were extracted for proteolytic activity from senescing leaves.

Since nitrate reductase activity was lost upon freezing, germinated wheat plants were also grown in a culture room in pots containing nutrient solution with 3.5 mM nitrate as nitrogen source (Thomas et al. 1979). The plants were grown in a 14 h light/10 h dark cycle. Leaves of 2–3 week-old seedlings were used as the source of nitrate reductase. These leaves were collected immediately before extraction.

Enzyme extraction and pre-incubation of extracts

Leaf samples were homogenized with a Polytron mixer (Kinematica, Luzern) for 20 s at low speed and for 5 s at full speed. Young leaves as source for glutamine synthetase and nitrate reductase activity were mixed in 4 volumes of 100 mM imidazole/HCl buffer pH 7.5 containing 15 mM $MgSO_4$, 12 mM mercaptoethanol and 1% w/v insoluble polyvinylpolypyrrolidone (PVPP). Young and senescing leaves as source for proteolytic activity were extracted in 4 and 10 volumes respectively of 25 mM imidazole/HCl buffer containing 1% w/v PVPP and 12 mM mercaptoethanol. Extracts were passed through Miracloth (Calbiochem., San Diego) and centrifuged for 15 min at 25,000× g. Supernatants were desalted by centrifugation through Sephadex G-25 (Feller et al. 1977) equilibrated with extraction medium except that PVPP and in the case of the extracts for proteolytic activity also mercaptoethanol was omitted.

Extracts from young leaves were mixed (1 : 1) with either buffer (25 mM imidazole/HCl pH 7.5) for controls or with the eluates from senescing leaves. The samples (pH 7.5) were pre-incubated in stoppered test tubes in a water bath at 30 °C.

Seperation of proteolytic activity on DEAE-cellulose

The washed DEAE-cellulose was equilibrated with 25 mM imidazole/HCl buffer pH 7.5. Glass columns were packed under pressure a day before use and stored at 4 °C. Before the desalted crude extract was loaded, the column (15 × 1.5 cm) was washed with buffer for one hour at a pumping speed of about 50 ml/h. After loading the extract the column was washed again with 30 ml buffer and then the bound protein was eluted with a linear gradient from 0 to 1 M NaCl in 250 ml elution buffer. The volume of the fractions collected was 3.3 ml. In the case of the step-elution, the elution buffer contained 0.4 M NaCl. Since the peak I-activity was not concentrated in the column, the combined fractions of peak I were concentrated 5–8 fold in a dialysis bag (Sigma, 250-9 U) kept on polyethyleneglycol MW 10,000 at 4 °C. The combined fractions of peak II were desalted on Sephadex G-25 columns equilibrated with 25 mM imidazole/HCl buffer pH 7.5 in order to remove the NaCl.

Enzyme assays

The proteolytic activity at pH 7.5 was determined with acetylated casein. The acetylation reaction was done according to Salgo and Feller (unpublished). Casein (2.5 g) was dissolved in 50 ml 100 mM imidazole/HCl buffer pH 7.5. During the acetylation procedure the solution was vigorously stirred at room temperature. Acetic anhydride (200 µl) was added and after 10 min the solution was readjusted to pH 7.5 with 10 N KOH (300–500 µl). This procedure was repeated 5 times at intervalls of 10 min. After filtration of the solution through Sephadex G-25 (Feller et al. 1977) the protein content was determined with the 'BIO-RAD' protein-reagent according to Bradford (1976). The stock solution of the acetylated casein was stored at –20 °C.

Extract (50 µl) and 100 µl substrate solution (0.5% acetylated casein (w/v) in 100 mM imidazole/HCl buffer pH 7.5) were mixed and incubated during 1 h at 30 °C for inactivation experiments and during 90 min at 37 °C for column elution profiles and pH optima. At the end of incubation 10 to 30 µl of the assay mixture were added to 200 µl TNBS-solution (17.5 mg trinitrobenzene sulfonic acid in 50 ml 0.5 M borate buffer pH 9.5). After 1 h the optical densities were read at 405 nm. A calibration curve was made with alanine from 0 to 50 nmol per well. For blanks the enzyme extract was added to the substrate solution immediately before measuring the free amino groups.

Glutamine synthetase was determined by measuring the ATP-dependent production of $\varnothing \rightarrow \gamma$-glutamylhydroxamate (Streit and Feller 1983).

Nitrate recuctase activity was measured as the NADH-dependent formation of nitrite (Streit and Feller 1982).

Results

Between 70 and 80% of the proteolytic activity loaded on the DEAE-cellulose column was recovered after chromatography of wheat leaf extracts at pH 7.5. Three major peaks were detected in the elution profile (Fig. 1). Peak I was not bound to the column under the conditions used and increased markedly during senescence. The other peaks (II A and II B) were found in the NaCl gradient. These peaks represented the main activity in young leaves and they also increased during senescence, but this increase was less distinct than that for peak I. The pH optimum for the hydrolysis of the acetylated casein by peak I was between 6 and 9, that for the hydrolysis by the peaks II A and II B between 4 and 7 (Fig. 2).

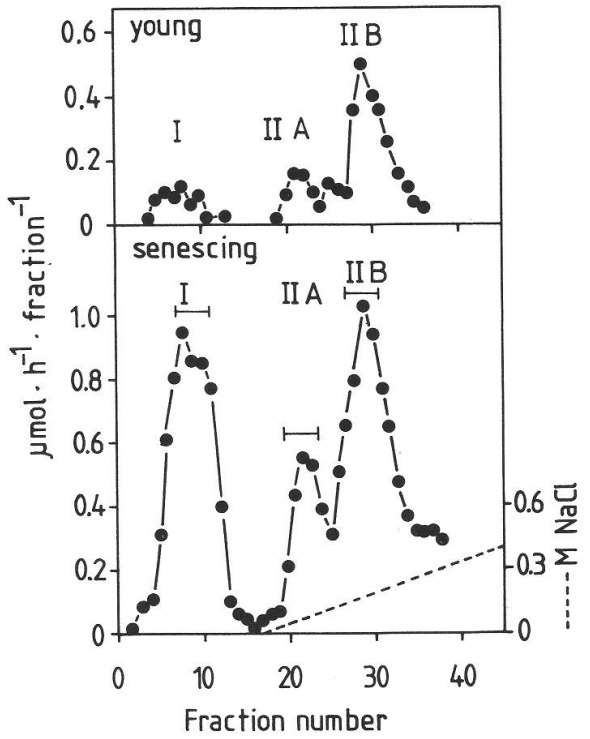

Fig. 1. DEAE-cellulose chromatography of proteolytic activities from young (A) and senescing (B) wheat leaves. The chromatograms were developed with a linear NaCl gradient from 0 to 1 M in 250 ml 25 mM imidazole/HCl pH 7.5. The fractions (3.3 ml) below the horizontal bars were combined for the analyses of the pH optima.

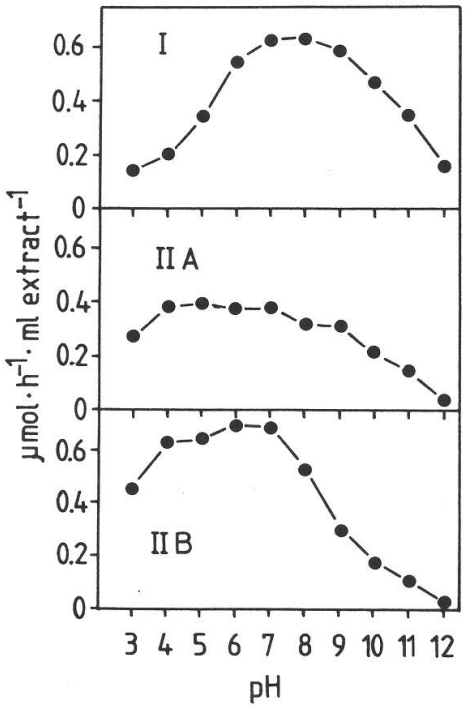

Fig. 2. Effect of pH on the hydrolysis of acetylated casein by the peptide hydrolases from senescing leaves. Peak I, II A and II B were seperated as shown in Fig. 1. One single buffer system was used for the whole pH range. It contained 25 mM phosphate, 25 mM acetate and 25 mM cyclohexylaminomethan sulfonic acid. The pH was adjusted with HCl or KOH.

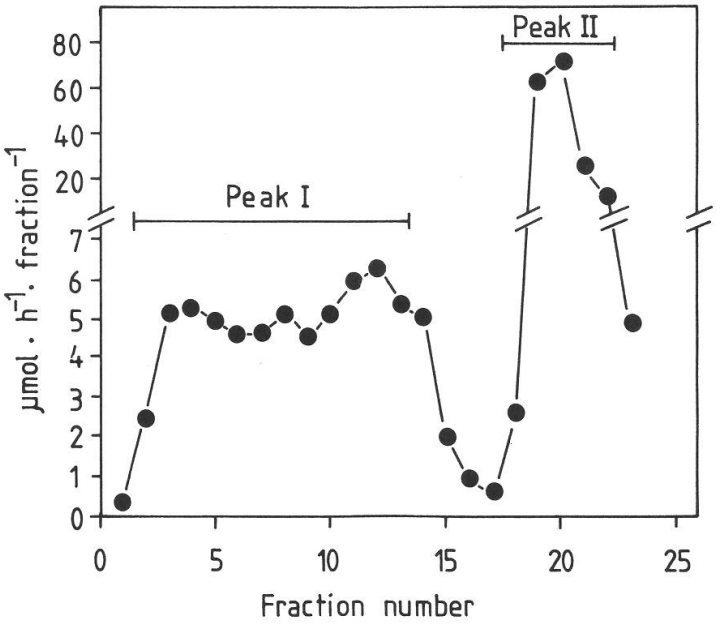

Fig. 3. Seperation of proteolytic activities from senescing wheat leaves for inactivation experiments. Peak I was eluted with starting buffer (25 mM imidazole/HCl pH 7.5). Peak II was eluted with starting buffer containing 0.4 M NaCl. The fractions (3.3 ml) below the horizontal bars were combined and prepared as described under materials and methods.

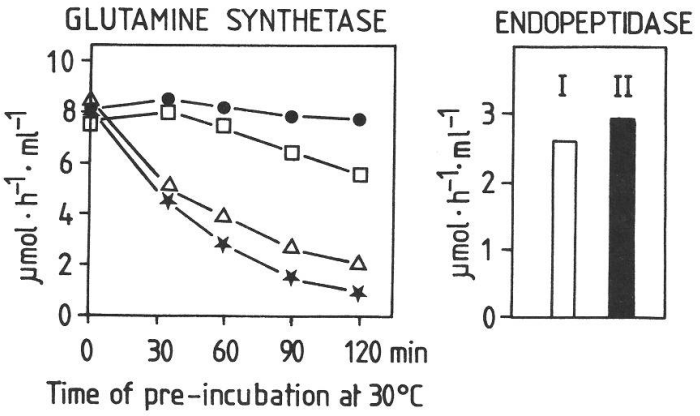

Fig. 4. Stability of glutamine synthetase in presence of peak I- and peak II-eluates. Extract from young leaves (0.3 ml) was mixed with: 0.3 ml buffer (●), 0.2 ml buffer +0.1 ml peak I-eluate (△), 0.2 ml buffer +0.1 ml peak II-eluate (□), 0.1 ml buffer +0.1 ml peak I-eluate +0.1 ml peak II-eluate (✱). The symbols for glutamine synthetase activity and the bars for endopeptidase activity represent the means of 4 seperate measurements. The endopeptidase activities in the eluates from peak I and II were measured before mixing with young leaf extract.

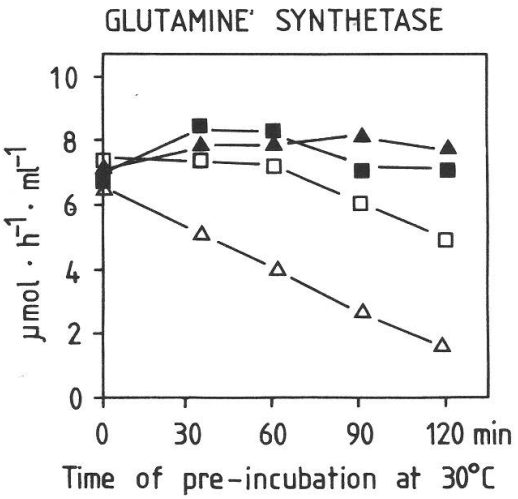

Fig. 5. Stability of glutamine synthetase in presence of native and heat denatured column eluates from peak I and II. Extract from young leaves (0.3 ml) was mixed with: 0.2 ml buffer +0.1 ml heated peak I-eluate (▲), 0.2 ml buffer +0.1 ml heated peak II-eluate (■), 0.1 ml buffer +0.1 ml peak I-eluate +0.1 ml heated peak II-eluate (△), 0.1 ml buffer +0.1 ml peak II-eluate +0.1 ml heated peak I-eluate (□). The symbols represent the means of 4 seperate pre-incubations.

Since peaks IIA and IIB behaved similarly during senescence only one fraction with proteolytic activity bound to the column (peak II) was prepared by step elution with 0.4 M NaCl in the elution buffer (Fig. 3). The inactivation of glutamine synthetase was delayed in the presence of 0.2 M NaCl (data not shown); therefore, the peak II-eluate was desalted in order to remove the NaCl. About 20% of the peak II-activity was lost by desalting on Sephadex G-25 columns. The effects of the peak I- and peak II-eluates from senescing leaves on the stability of glutamine synthetase from young

leaves were considerably different (Fig. 4). The addition of peak I-eluate to young leaf extract caused a considerably more rapid inactivation of glutamine synthetase than the addition of peak II-eluate. When the eluates from peak I and II were mixed, no interferences were detected (Fig. 4). Heated (5 min at 96 °C) eluates from peak I and II were ineffective (Fig. 5). The inactivation of glutamine synthetase caused by peak I was not delayed with heated peak II-eluate. Similarly, heated peak I-eluate had no effect on the inactivation of glutamine synthetase by peak II.

Nitrate reductase was much less stable than glutamine synthetase under the same conditions (Fig. 4 and 6). Like glutamine synthetase, nitrate reductase was more rapidly inactivated by peak I than by peak II. A considerable portion of nitrate reductase activity was already lost during the 3 min between mixing young leaf extract with peak I and the start of the initial enzyme assays (Fig. 6).

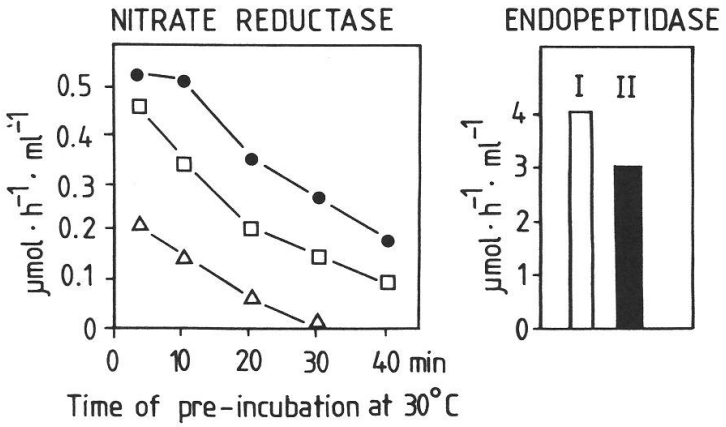

Fig. 6. Stability of nitrate reductase in presence of peak I- and peak II-eluates. Extract from young leaves (0.2 ml) was mixed with: 0.2 ml buffer (●), 0.2 ml peak I-eluate (△), 0.2 ml peak II-eluate (□). The symbols for nitrate reductase activity represent the means of 3 seperate pre-incubations. The endopeptidase activites in the eluates from peak I and II (duplicate measurements) were determined before mixing with young leaf extract.

Discussion

During senescence of wheat leaves the endopeptidase pattern changed markedly in favor of peak I, which had a pH optimum in the alkaline range. This change can explain the shift in the pH optimum observed for total proteolytic activity (Martin and Thimann 1972, Feller et al. 1977, Thomas 1978).

The results concerning the inactivation of glutamine synthetase and nitrate reductase are consistent with the hypothesis that the enzymes are inactivated by proteolytic attack as supposed earlier (Wray and Kirk 1981, Streit and Feller 1982, Batt and Wallace 1983). However, peak I-eluate was much more effective than that of peak II, although the proteolytic activities against acetylated casein were similar. Therefore, the degredation of enzyme proteins may depend not only on the quantity, but also on the patterns of the endopeptidases present.

The bulk of the endopeptidase activities was found in the vacuoles (Boller and Kende 1979, Heck et al. 1981, Lin and Wittenbach 1981). It remains open, whether or not the activities mentioned above play a major role in the mobilization of leaf proteins during senescence. Recent observations on chloroplast degradation suggest that proteins are degraded within the organelles and that compartmentation is maintained until late in senescence (Martinoia et al. 1983, Mae et al. 1984). Some proteolytic activity was found to be associated with the chloroplast and these peptide hydrolases could initiate the degredation of proteins in this compartment (Dalling et al. 1983).

Our results suggest that the various leaf endopeptidases differ in their relative activities against exogenous substrates (e.g. casein) and endogenous enzyme proteins. Therefore, the pattern of the peptide hydrolases present and the susceptibilities of the substrate proteins should be taken into account when investigating proteolysis in a particular compartment.

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