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ACTIVITIES AND MULTIPLICITY OF PHENOLASE FROM SPINACH CHLOROPLASTS DURING LEAF AGEING

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; phenolase; chloroplast; multiple forms; senescence.

Abstract—Phenolase activity in spinach leaves homogenates depends on the stage of development of leaves and on the kind of homogenization procedure. Under constant experimental conditions it is low in non-senescent leaves. With the onset of senescence there is a 15–20-fold increase in soluble activity in the supernatants of broken chloroplasts as well as an increase in activation of latent phenolase in fractions containing thylakoids. This rise in activity is due to an increase in particular multiple forms, differing for supernatants and membrane sediments. Phenolase from spinach lacks monophenolase and laccase activities.

INTRODUCTION

Plant phenolase (EC 1.14.18.1), recently reviewed by Mayer and Harel [1], in spinach leaves is located in the chloroplasts [2, 3]. Latent forms can be activated by storage of membrane-containing fractions [3, 4], frost [4] and various detergents [3, 5]. *In vivo* activity seems to be very low or even non-existent [2, 3]. Satô and Hasegawa reported seasonal changes in phenolase activity from spinach [5]. Changing, mostly increasing activities during leaf senescence (e.g. [6]) and after injury or infection (e.g. [7]) are known from other plants. This paper describes strong increase of phenolase activity and changes of electrophoretic phenolase pattern during spinach leaves senescence. Up to now, it is not clear if these changes are due to increased synthesis of enzyme. Alternatively, latency of pre-existing phenolase could be changed by senescence, resulting in higher rates of activation of latent enzyme during homogenization and extraction procedures. This assumption is favoured by the fact that spinach leaf phenolase is rapidly activated during homogenization and fractionation of cells [3] and by some additional findings presented in this paper.

RESULTS

Influence of homogenization

The initial activity of phenolase in homogenates from one batch greatly depends on the experimental conditions (Table 1). Under conditions which give high yields of morphologically and functionally intact chloroplasts (e.g. high concentrations of sucrose), phenolase activities remain low. High salt concentrations, which cause loss of the chloroplast envelope [8], and inorganic buffer systems lead to high initial rates of activity.

Phenolase activities during leaf aging

In order to compare enzyme activities from different developmental stages, differences in the amount of intact chloroplasts in the homogenates must be avoided. Constant experimental conditions were maintained by using the same blender and one hypotonic medium in all cases. According to light microscopic investigations, all homogenates contained broken chloroplasts and slightly swollen thylakoids. Intact chloroplasts were not chosen for the experiments because those from senescent leaves are not stable and will disintegrate even in hypertonic isolation media.

Constant time intervals between the steps of all preparations were strictly followed, because membrane-containing fractions showed a rapid time-dependent activation, which was due to liberation of bound latent forms [3, 9]. Homogenates H, membrane-free supernatants S and membrane-containing fractions P were prepared (for details see Experimental). The initial phenolase activities were estimated exactly 70 min after onset of homogenization (Fig. 1). Activation rates during 54 hr of storage of separated membrane fractions in an ice bath are demonstrated in Fig. 2 for four representative experiments. Activation is restricted to membrane-containing fractions. Initial activities and activation are highest during senescence (5 and 7 weeks), but lower before (1–3 weeks) and after (9 weeks) onset of senescence (see below). Initial activities in the supernatants are nearly as high as those from the corresponding freshly prepared homogenates (Fig. 1). The sum of activities of P and S is higher than the homogenate activities (see Discussion). From Fig. 2 it can be concluded that differences of initial activities at different leaf ages indicate differences of activation rather than increased contents of active enzyme.

There is a striking increase of phenolase activities after the third week, reaching a peak around week 7 and followed by a decrease in the last 2 weeks (Fig. 1). The life cycle of primary leaves is complete 9 weeks after the beginning of their unfolding, when they become shrivelled and turn yellowish to brown in colour. In some preparations, decline of activity had already started in

Table 1. Effects of different grinding procedures on phenolase activities of freshly prepared homogenates

Blender* speed	Grinding medium: Buffer†	Grinding medium: Additions	Relative initial phenolase activity (%)
Low	A	27 mM NaCl	100
High	A	27 mM NaCl	140
Low	A	600 mM sucrose + 27 mM NaCl	77
High	A	600 mM sucrose + 27 mM NaCl	154
Low	A	327 mM NaCl	174
Low	B	none	170
Low	B	600 mM sucrose	142

Freshly harvested, non-senescent spinach leaves from one batch were homogenized in different media. Phenolase activity was estimated polarographically at 25° in darkened cuvettes exactly 10 min after onset of homogenization. The reaction mixture contained 2.8 ml of air-saturated 67 mM phosphate buffer, pH 6.8; 0.1 ml substrate solution (final concentration: 7.5 mM 4-methyl-catechol) and 0.1 ml homogenate containing 250–300 μg chlorophyll/ml.

*Blender: low speed = 800 rpm (Braun Multimix), high speed = 45,000 rpm (Virtis Homogenizer).

†A = 10 mM tricine–NaOH, B = 10 mM $Na_4P_2O_7$–HCl.

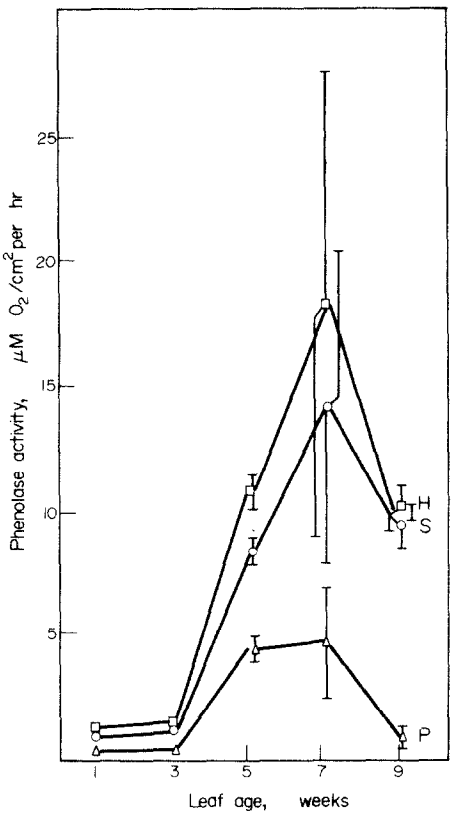

Fig. 1. Initial phenolase activities of homogenates (H), membrane pellets (P) and supernatants (S) from spinach primary leaves of different ages. Activities were estimated 70 min after onset of homogenization as described in Table 1 and calculated on a leaf area base. Age of leaves: weeks from the beginning of unfolding of primary leaves. ——, s.d. from six single estimations from plants of two separate sowings.

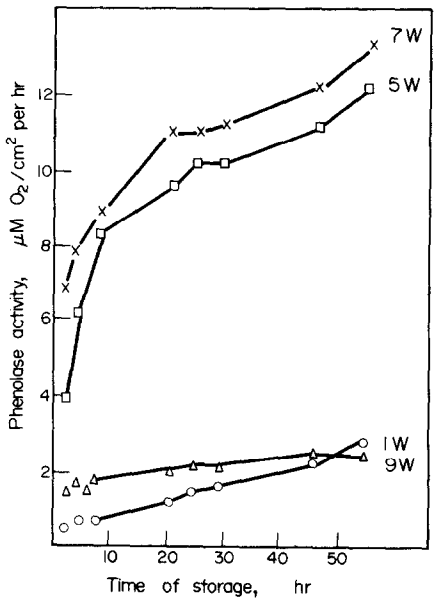

Fig. 2. Activation of latent phenolase from spinach primary leaves of different ages during storage of fractions P at 0°. Estimation of phenolase activity and material is described in Fig. 1. Aliquots (0.1 ml) of the stored suspensions of membrane pellets (P) were taken for estimation of phenolase activity. Leaves were harvested 1, 5, 7 and 9 weeks after the beginning of their unfolding from different plants to avoid injury effects.

7-week-old leaves leading to great variabilities in phenolase activity at this stage of development. The increase in phenolase activity is even more pronounced when calculated on a chlorophyll, protein or fresh weight base. Experiments have been repeated several-fold with plants from separate sowings. Absolute magnitude of activities and duration of leaf development reveal great variation, but in all cases so far investigated there is the same sequence of changes in phenolase activity for the plants of one plot.

Correlation between phenolase activity and senescence

The onset of senescence is marked by a decrease in chlorophyll content after the third week (Table 2c), and by a more pronounced loss of protein, membrane protein, e.g. from chloroplasts as well as soluble protein (Table 2a, b). There is a decrease in photosynthetic activities calculated per unit of chlorophyll, which started at the same time (Table 2d, e). This means the inactivation of both photosystems is not only due to the loss of chlorophyll per unit leaf area, but also to a breakdown of the functional integrity of the thylakoid membrane. The increase of phenolase activity (Figs. 1 and 2) starts at the same time as the disintegration of the chloroplasts after the third week of leaf development. The correlation of the rise in phenolase activity with those parameters which indicate senescence has been studied. To get high levels of significance, the log activities have been used because phenolase activity reveals nearly a logarithmic increase during leaf ageing. As indicated in Table 3 there is no correlation between phenolase and dry wt or fresh wt respectively, but a strong negative correlation between phenolase activity and both photosynthetic activities, as well as chlorophyll and protein contents. Thus the loss of thylakoid membrane material and photosynthetic activities in senescing leaves result in a logarithmic (non-linear) increase in activities of both soluble phenolase in the homogenate supernatants and higher rates of activation of the latent enzyme in the membrane pellets.

Changes of electrophoretic patterns of phenolase

Newly appearing phenolase forms during senescence could indicate *de novo* synthesis. Thus changes of electrophoretic patterns have been investigated. As we have reported earlier, ten proteins which reveal phenolase activity can be separated by disc-electrophoresis [3]. All of them can be derived from isolated chloroplasts. There are no proteolytic degradation products, since no proteolytic activity has been found in isolated chloroplasts (unpublished results).

Table 2. Protein and chlorophyll contents and photosynthetic activities of primary leaves of different ages

Leaf age (weeks)	Protein Homogenate (mg/cm^2)	Protein Fraction P (mg/cm^2)	Chlorophyll (Fraction P) ($\mu g/cm^2$)	PS I (Fraction P) ($\mu M\ O_2/hr/mg\ chl$)	PS I+II (Fraction P) ($\mu M\ O_2/hr/mg\ chl$)
1	0.66 ± 0.05	0.25 ± 0.01	25.2 ± 0.8	678	102
3	0.54 ± 0.07	0.19 ± 0.01	28.0 ± 1.6	703	73
5	0.23 ± 0.06	0.12 ± 0.03	19.1 ± 3.8	453	43
7	0.09 ± 0.04	0.05 ± 0.02	5.4 ± 2.1	404	13
9	0.08 ± 0.03	0.03 ± 0.01	0.4 ± 0.1	116	0

Protein and chlorophyll are estimated in the homogenates (H) and in the resuspended membrane pellets (P). Chlorophyll contents of P and the corresponding homogenates are identical. Results are calculated on a leaf area base. Data refer to four single estimations from plants of two separate sowings. Photosynthetic activities of P are given for one representative plot of plants and are calculated on a chlorophyll base. Photosystems (PS) I and I + II are estimated uncoupled according to ref. [23] with some modifications in a polarographic assay system at pH 8.0 and 25°. Final concentration: 200 mM sucrose; 3.3 mM $MgCl_2$; 15 mM tricine-NaOH buffer; 10 mM methylamine. Additions for PS I: 50 μM DCMU; 1 mM methylviologen; 7 mM Na ascorbate; 100 μM DCPIP. Additions for PS I + II: 4 mM $K_3[Fe(CN)_6]$.

Table 3. Correlation between phenolase activities and several reference units (protein, chlorophyll, fr. wt, dry wt, photosynthetic activities) during senescence of spinach primary leaves

Reference unit	n	$r_{(S)}$	sig. lev. (%)	$r_{(P)}$	sig. lev. (%)
Protein content	14	−0.928	99.9	−0.661	99.0
Chlorophyll content	14	−0.787	99.9	−0.496	90.0
Fr. wt	14	−0.090	not sig.	−0.363	not sig.
Dry wt	14	−0.294	not sig.	−0.147	not sig.
PS I activity	14	−0.896	99.9	−0.801	99.9
PS I + II activity	14	−0.949	99.9	−0.832	99.9

Phenolase activities in supernatants (S) and membrane pellets (P) of 1,3,5,7 and 9-week-old leaves (Fig. 1) from two separate sowings were used for this calculation. Total number of estimations (n) was 14. The correlation coefficients (r) were calculated from the logarithms of phenolase activities in fractions P and S and the respective reference units of each leaf age (Table 2 or unpublished data). Phenolase activities and the various reference units were estimated on a leaf area base.

PS = photosystem; sig. lev. = significance level.

In order to discover if real catecholase activity is increased during senescence, browning reactions were stydied after incubation of electrophoresis gels with inhibitors and several substrates. Forms III–XI (Fig. 3) from any stage of development do not reveal any monophenolase or laccase activities. With 4-methylcatechol or DOPA as substrates, they are not inhibited by catalase, but are strongly inhibited by 1 mM cyanide or diethyldithiocarbamate. Thus they are catecholases. In contrast, forms I and II are not inhibited by cyanide and they convert monophenols (*p*-coumaric acid, *p*-cresol, DL-tyrosine) and *p*-diphenols (hydroquinone, *p*-phenylenediamine) to brown reaction products.

For interpretation of changes in phenolase pattern in Fig. 3 it should be mentioned that two groups of bands have shown well distinguishable behaviour during extensive experiments published earlier [3, 4]: group A ('stromatic' forms VIII–X) and group B ('membrane' forms III–VI). Both groups are found in supernatants of homogenates, but only group B in membrane sediments from non-senescent leaves.

Fig. 3 shows that very young leaves contain only 'membrane' forms (group B). Later on, 'stromatic' forms (A) appear in the supernatants only. During senescence there is a striking increase of 'stromatic' forms, especially of form X, in the supernatants, and an increased zone of smear in the membrane pellets expanding from form VI to IX. In fully senescent leaves, which do not contain intact chloroplast membranes anymore, only form XI is found. From co-electrophoresis it can be concluded that XI is different from X.

We have tried to determine whether phenolase forms can be converted artificially *in vitro*. For conversion the following agents have been used: 4 M urea, acid shock pH 3.5 (according to ref. [10]), alkali-shock pH 11.5 and extraction with acetone and ether to remove lipid material. SDS-electrophoresis cannot be performed because phenolase is totally inhibited during the run. In no case at any leaf age could any *in vitro* conversion be detected by electrophoresis.

Phenolase from secondary leaves

The reported changes in activity and multiplicity are not restricted to primary leaves. As can be seen in Table 4.

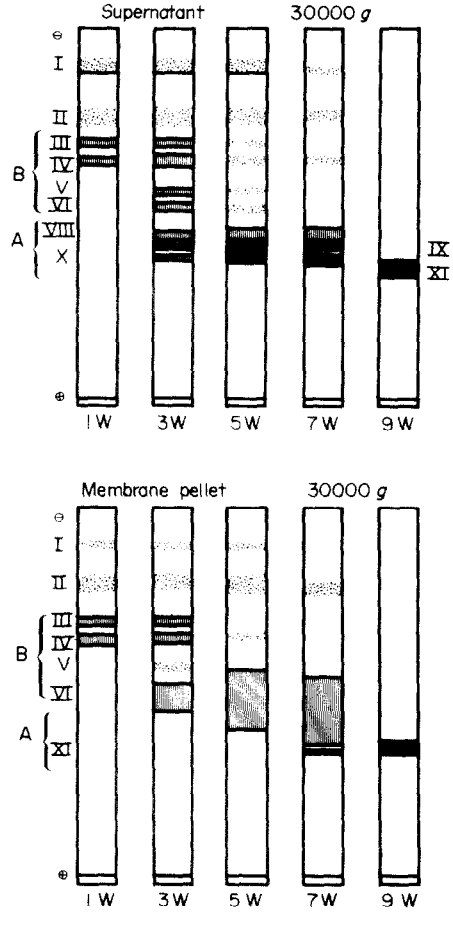

Fig. 3. Gel electrophoretic pattern of the multiple forms of phenolase in S and P from spinach primary leaves of different ages. Fractions S and P were stored for 12 hr at 0° and centrifuged 1 hr at 30000 g. The resultant supernatants were concentrated by ultrafiltration, centrifuged again (1 hr, 30000 g) and 10–300 μg of protein were applied to each column. Multiple forms were detected by incubation of the gels in 1 mg/ml L-DOPA as substrate. Age of leaves: as described in Fig. 1; W = weeks.

Table 4. Phenolase activities of spinach secondary leaves

Position of leaves at the stem	Phenolase activity (μM O_2/hr/mg fr. wt)	
	Fraction S	Fraction P
Top	138	52
Middle	630	240
Base	950	565

Secondary leaves from one plot of mature spinach plants were harvested at the same time. Two upper (full green) leaves, two at the middle (light green) and two leaves at the base of each plant (yellow) were collected. Preparation of fractions S and P and phenolase assay are described under Experimental and in Table 1. Leaf area was not determined.

the younger leaves from the top of mature plants reveal less activity than the older leaves down the stem. Additionally, the electrophoretic pattern of younger and older secondary leaves investigated at the same time show principally the same changes as primary leaves of different ages. i.e. an enrichment of 'stromatic' forms in the supernatants of older leaves and a broadening of the zones of 'membrane' bands in the pellets. Minor differences are due to the fact that not exactly the same developmental stages of leaves were used for the experiments described in Figs. 3 and 4. Overall activity, however, was different in secondary and primary leaves respectively. Calculated on a fr. wt base maximum activity of 1000 μM O_2/hr/mg is attained with secondary leaves, whereas old primary leaves reveal not more than 500 μM O_2/hr/mg, corresponding to 22 μM O_2/hr/cm^2 in Fig. 1.

DISCUSSION

Phenolase activity rises considerably during the life span of spinach leaves. Similar increase of activity has been reported from other plants, e.g. rice [6] and apple [11], but its biochemical mechanisms have not been elucidated so far. In contrast to spinach, 8 other species recently investigated revealed no strong correlation between onset of senescence and rise in phenolase activity [12].

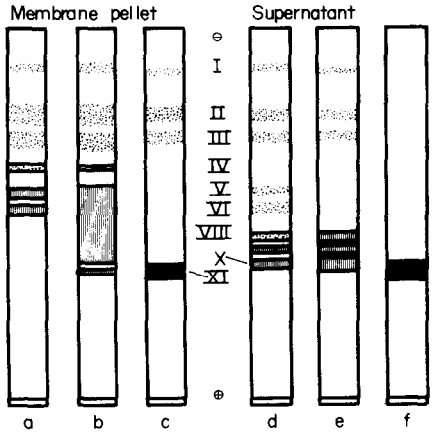

Fig. 4. Multiple forms of phenolase from secondary leaves. Samples from the experiment described in Table 4 were taken. Electrophoresis and preparation of samples are described in Fig. 3 and Experimental. Protein (300 μg) was applied to each column. Position of leaves at the stem: a,d = top; b,e = middle part; c,f = base.

Phenolase activity as measured in homogenates strongly depends on the maintenance of thylakoid integrity and thus on homogenization procedure. It has been shown by Lieberei and Biehl [3] that in young spinach leaves phenolase activity is near to zero, but spontaneous activation starts by leaf homogenization and continues during storage of homogenate fractions. This activation is due to release from the membranes of thylakoid-bound latent phenolase. The enzyme released from the membranes during storage reveal group B bands ('membrane' forms) only by disc-electrophoresis and clearly can be distinguished from group A bands ('stromatic forms') which are found in stroma containing fractions of chloroplasts only [3, 4]. All soluble phenolase forms slightly inactivate during *in vitro* ageing, thus leading to decreased S/P ratios of phenolase activity.

The summarized activities in fractions S and P exceed that one in the homogenate (see text). This behaviour is not due to the presence of soluble inhibitors in the leaf brei but to the rapid activation of membrane-bound latent phenolase upon resuspension of the membrane pellet. The same results (higher activities in supernatant plus resuspended pellet versus unfractionated suspension) is obtained when suspensions of isolated, several times washed thylakoid membranes are fractionated by centrifugation [3, 9].

As shown in Figs. 1 and 2, increase of initial homogenate activity during onset of senescence is an expression of facilitated activation rather than different steady-state activities. This *in vivo* acceleration of activation is similar to the increased activation *in vitro* by thylakoid swelling procedures (e.g. [13]). It can be correlated (Table 3) to the early loss of structural integrity of thylakoid membranes, accompanied by the loss of photosynthetic activities [14] during onset of senescence.

It is highly interesting that this correlation is valid not only for the 'membrane' forms ($r_{(P)}$ in Table 3) but also for the 'stromatic' forms ($r_{(S)}$ in Table 3), indicating that both groups of phenolase forms are derived from the thylakoids, the 'stromatic' forms being spontaneously released during homogenization in the hypotonic medium. In fully senescent leaves, which have lost most of their membrane material and which contain only 'stromatic' forms, high activity in fraction S but low continuous activation in fraction P is found.

The high correlation of phenolase activities in both fractions S and P to senescence and the observed changes in the pattern of multiple forms may imply that latent membrane-bound phenolase is converted to soluble 'membrane' forms in young leaves, but is converted more and more to 'stromatic' forms in the course of thylakoid modification during senescence. Thus, in the course of *in vivo* ageing, form X, in particular, increases as does the ratio of activities S/P (Fig. 1).

Though ripening of fruits or storage organs, e.g. sugar beets, cannot be paralleled to leaf senescence, there is a striking coincidence: a rise in phenolase activity in the soluble versus the particulate fraction in apples [15], olives [16] and sugar beet [17]. Just the opposite behaviour is found in peaches [18] and in contrast to

senescence of spinach leaves, total activity drops during ripening of fruits and beets, respectively [15–18].

Interconvertibility of phenolase forms has been demonstrated for several plants [1, 10] and for acetone preparations of spinach [5]. Our own attempts to convert spinach phenolase forms separated by electrophoresis from native homogenate fractions were unsuccessful (see text and ref. [9]) but serological identity of 'membrane' and 'stromatic' forms in spinach leaves has been reported [19], allowing us to assume interconversion of forms during *in vivo* senescence. Thus increased phenolase activity due to the appearance of special multiple forms (VI, X) does not require *de novo* synthesis but can be a result of accelerated activation of pre-existing latent enzyme, which undergoes a 'transition' of group A to group B forms *in vivo*. The biochemical basis of the differences between the special phenolase bands is not entirely clear up to now [4].

Latency, pattern and activity of multiple phenolase forms are controlled by the physical and chemical integrity of thylakoid membranes. Phenolase, as a membrane protein, may associate with other membrane constituents, which change or self assemble during senescence and during its release from the thylakoids.

EXPERIMENTAL

Spinach plants (*Spinacia oleracea* L. cv Matador) were field-grown from March to July. Primary leaves were harvested, washed twice and homogenized (10 sec × 3) with a blender (Braun Multimix) in 2 n ml per n g fr. wt of a grinding medium containing 27 mM NaCl, 1 mM $MgCl_2$ and 10 mM tricine–NaOH buffer, pH 7.8. The homogenates were filtered through 4 layers of cotton gauze and centrifuged 1 min at 200 g to remove cell debris. The resultant cell-free homogenates (fractions H) were submitted to centrifugation (1 hr, 30 000 g) to obtain membrane-free supernatants (S) with chlorophyll contents below 1 μg/ml. Centrifugation started within 5 min after rupture of cells. The membrane pellets were resuspended in fresh grinding medium to obtain fractions P and were adjusted to the vols of the corresponding fractions S. All steps were done at 0–4°. Fractions were kept in the dark in an ice-bath and constant time intervals between all steps of parallel investigations were strictly followed. Protein determination was performed with a modified Biuret reaction according to ref. [20] with the addition of 1% Triton X-100 to dissolve membrane proteins. Chlorophyll was estimated in 80% aq. Me_2CO according to ref. [21]. Electrophoresis was performed according to ref. [22] with Tris–HCl buffers, pH 8.9 and 6.7 respectively, for the 7% acrylamide gels. Multiple forms were detected by incubation of the gels in 1 mg/ml substrate soln in O_2-saturated 67 mM K/NaPi buffer, pH 6 at 25° according to ref. [4]. For inhibitory studies the gels were pre-incubated for 30 min with 10 U/ml catalase or 10 mM inhibitor, followed by incubation in the substrate soln, with the indicated concn of inhibitor. For conversion studies the concentrated phenolase-containing fractions were treated with urea or pH shocks according to ref. [10] or washed with Me_2CO and Et_2O and redissolved in H_2O. For preparation of samples see Fig. 3. Phenolase assay is described in Table 1. All values were corrected for auto-oxidation of substrates. In each experiment fr. wt per leaf area, chlorophyll per fr. wt and chl. per ml fraction were estimated, assuming the chl./phenolase ratio to be identical in the homogenate and the total leaf brei. Chl. content of the corresponding membrane pellets was used to calculate phenolase activity per leaf area in the chlorophyll-free supernatants.

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