Phytochemistry, Vol. 20, No. 5, pp. 955–959, 1981.
Printed in Great Britain.

0031–9422/81/050955–05 $02.00/0

ACTIVATION OF LATENT PHENOLASE DURING SPINACH LEAF SENESCENCE*

HANS-U. MEYER and BÖLE BIEHL

Botanical Institute of the Technical University of Braunschweig, Humboldtstr. 1, D-3300 Braunschweig, West Germany

(*Revised received* 11 *September* 1980)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; phenolase; latency; activation; multiple forms; *de novo* synthesis; senescence.

Abstract—The observed increase of phenolase activity and of its rate of activation during spinach leaf senescence is due to reduced binding of latent phenolase to the thylakoid membranes and not to *de novo* synthesis. The same amount of phenolase which is active in isolated thylakoid membranes from senescent leaves can be found in the membranes of non-senescent leaves after activation of latent enzyme. Tracer experiments give evidence that one multiple form which is responsible for the bulk activity in senescent leaves, is synthesized before, but not after the onset of senescence, indicating that pre-existing latent phenolase is converted to easily activating forms.

INTRODUCTION

Phenolase (EC 1.14.18.1) from spinach chloroplasts shows a 15 to 20-fold increase of activity during leaf senescence. This increase in both supernatants and membrane pellets from homogenates is accompanied by an increased dominance of at least two multiple forms [1]. Thus enhanced phenolase activity during leaf senescence may be related to *de novo* synthesis; but activation of latent phenolase, which has often been observed *in vitro* [2–5] may be an alternative explanation. The experiments described in this paper will give evidence that increase in phenolase activity during leaf senescence as measured in homogenates is not related to *de novo* synthesis but to enhanced activation of pre-existing latent membrane-bound enzyme forms.

RESULTS

Activation during storage and washing

In thylakoid membrane-containing fractions from spinach primary leaves of different ages, latent phenolase is spontaneously activated during *in vitro* storage [1]. From a series of such investigations, activation rates were extrapolated (Fig. 1). The only leaves which reveal high rates of phenolase activation are those where senescence has begun, as marked by a decline in chlorophyll content per leaf area [1]. In both young and fully senescent leaves spontaneous activation is low.

Prolonged storage of membrane-containing fractions at 0° did not activate phenolase to a steady state of activity. Washing (centrifugation and resuspension in fresh medium) caused further activation by solubilizing bound latent enzyme [6]. In order to activate almost all extractable phenolase without solubilizing the membranes, a process of washing, freezing and thawing of membrane-containing fractions (P)† was repeated 40 times. In this experiment (Table 1(a)) the overall activities from fraction P after 54 hr of storage and 40 washings, plus the small residue of activity of the ultimate membrane pellets were higher than the activity of unwashed P-suspensions (Table 1(a)). The difference is high, especially

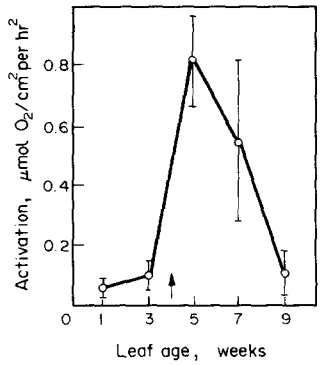

Fig. 1. Activation of latent phenolase during storage of resuspended membrane pellets. Freshly prepared membrane pellets from homogenates (P) from leaves of different ages were stored in an ice bath. Activation rates (i.e. increase in activity per hr) were extrapolated from the activity changes during the first 4 hr of storage. Activities were estimated polarographically at 25° in 0.067 M phosphate buffer pH 6.8 with 7.5 M 4-methylcatechol and calculated on a leaf area base. Leaf ages were estimated from the beginning of unfolding of primary leaves. →: onset of senescence, ⊢⊣: s.d. from 2 single estimations with plants of 2 separate sowings. In all cases investigated, there is the same sequence of changes of phenolase activity during senescence. Large s.d. applies to variation in duration of separate sowings. Especially after 7 weeks decline of activity has started in one sowing but not in the plants of the other one.

* This paper contains part of the thesis of H.-U. Meyer [16].

† "P" signifies the membrane pellet obtained by centrifugation (1 hr, 30000 g) of a hypotonic leaf homogenate. "S" signifies the resultant supernatant.

Table 1. Activity and extractability of phenolase from membrane sediments

Leaf age (weeks)	Activity of P, stored 54 hr, 0° (μmol/hr/cm²) (a)	(b)	% decline of activity in successive washings (c)
1, 3	1.52 ± 0.98	6.88 ± 3.12	24.9 ± 1.5
5, 7	10.74 ± 3.52	23.66 ± 5.86	27.6 ± 0.3
9	1.75 ± 0.97	2.11 ± 0.85	69.8 ± 4.4

Membranes (P) were freshly isolated from homogenates and resuspended in fresh medium. These suspensions were stored in an ice bath for 54 hr. After storage the suspensions were treated 40 times in the following way: they were frozen (−18°) and thawed twice, membranes were separated by centrifugation (1 hr, 30000 *g*) and the pellets were resuspended with fresh 0.067 M phosphate buffer pH 6.8 (= one "washing"). The volume of each washing corresponds to the volume of the initial homogenate supernatant (S). After 40 washings activities of the resuspended membrane sediments had lessened nearly to zero. Ages of leaves and estimation of phenolase activity as described in Fig. 1. Data from 1 and 3 and from 5 and 7 week old leaves are summarized.

(a) Activity of untreated 54 hr stored fractions P.

(b) Summarized activities of all successive washings of P plus activity of ultimate membrane pellet.

(c) Decline of activity between each two successive washings calculated by means of regression analysis.

Results from 4 experiments with plants of two separate sowings are summarized in the table.

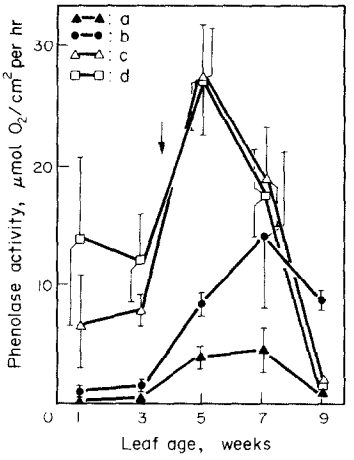

Fig. 2. Activation of latent phenolase by repeated washings of membranes and by SDS. Determination of activities, calculation and age of leaves are as described in Fig. 1. a: fractions P, 70 min after onset of homogenisation. b: fractions S, 70 min after onset of homogenisation. c: fractions P, overall activities after 54 hr of storage and 40 washings as described in Table 1. d: as in c, but additionally the membrane-free washings and the ultimate pellets were incubated 1 hr in 0.1% SDS at 0° before the assay of phenolase activity. →: onset of senescence. ⊢⊣: s.d. from 2 single estimations with plants of 2 separate sowings (for explanations see Fig. 1).

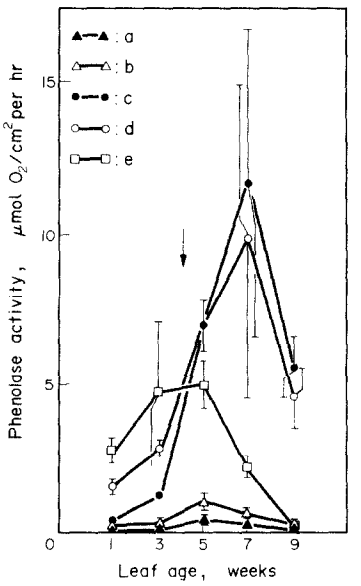

Fig. 3. Effects of frost and SDS on phenolase activity in supernatants and resuspended membranes from homogenates. Determination of activities, calculation and age of leaves are as described in Figure 1. a: fractions P, once washed after 54 hr of storage at 0° and resuspended in fresh grinding medium. b: as in a, but additionally the fractions were frozen (−18°) and thawed twice. Phenolase activity was estimated without further centrifugation in the membrane suspension. e: as in a, but additionally the fractions were incubated 1 hr with 0.1% SDS at 0°. Phenolase activity was estimated without centrifugation in the membrane suspension. c: fractions S, stored 55 hr at 0°. d: fractions S, stored 54 hr at 0° and additionally incubated 1 hr at 0° in the presence of 0.1% SDS. →: onset of senescence. ⊢⊣: s.d. from 2 single estimations from plants of 2 separate sowings (for explanation see Fig. 1).

in young leaves; but it is low in fully senescent leaves. The activity of each of the 40 successive washings was lower than that of the preceeding one. They declined to zero with first order kinetics.

After 54 hr of storage of unwashed membrane suspension P (Table 1(a)) and after 40 washings (Table 1(b)) the activities are much higher in 5- to 7-week-old senescing leaves than in non-senescent (1 or 3 weeks) or fully senescent leaves, whereas extractability (Table 1(c)) is highest in fully senescent ones. Thus extractability of phenolase by washing does not depend on the absolute amount of initial or extractable activities.

It is essential to note that not only activity but also activation and extractability of latent phenolase changes considerably during leaf senescence. This behaviour can be understood by taking into account drastic changes of multiple forms in the course of *in vivo* ageing [1] (see Discussion).

SDS-activation

The high rate of *in vitro* activation by washing in 5-week-old leaves favours the assumption that increase of phenolase activity during onset of senescence is due to changes of the molecular status of the enzyme but not to *de novo* synthesis. If this is true, non-senescent leaves

should contain sufficient latent phenolase to account for the high activities in senescent leaves. Storage, frost and washings do not solubilize membranes. Possibly thylakoid membranes contain inactive phenolase which can be activated only by total solubilisation of the membranes. SDS (sodium dodecylsulfate) destroys membrane architecture (e.g. [7]) and it causes highest activation rates [8]. On the other hand SDS inactivates isolated (active) phenolase (see below). Therefore we used SDS in two different experiments (Figs. 2 and 3). In the first, activities at different leaf ages were compared without *in vitro* activation (Fig. 2, a and b, fractions P and S), after activation by storage and washing of P as described in Fig. 1 (Fig. 2(c), sum of overall activities), and after additional incubation of all washings in 0.1 % SDS for 1 hr at 0° (Fig. 2(d). Two conclusions can be drawn from Fig. 2:

1. The low activity in non-senescent leaves (Fig. 2 a and b, total of P + S activities at 1–3 weeks) can be increased by washings and SDS-treatment of the original fraction P (Fig. 2(d) at 1–3 weeks) to an amount which is nearly as high as the overall activity of the untreated fractions P + S from senescing leaves (Fig. 2, a and b, total of P + S at 5–7 weeks) indicating the same activity potential.
2. In non-senescent leaves SDS causes an activation additional to that obtained by washing and frost, whereas in senescing leaves it does not (Fig. 2, c and d). Thus young leaves contain phenolase which can be activated by dissociating agents only.

The second experiment gives additional information: SDS does not activate bound phenolase only (by membrane disintegration) (Fig. 3, a and e) but activates non-membrane-bound phenolase as well (Fig. 3, c and d). However, SDS activation of soluble phenolase is found only in supernatants of non-senescent leaves (Fig. 3(d), 1–3 weeks). In contrast, activity of soluble phenolase from senescing leaves (Fig. 3(d), 5–7 weeks) is not increased by SDS or is even reduced. In this experiment, fractions were incubated for 1 hr at 0° in 0.1 % SDS. In order to understand the effect of SDS, the time-dependent change of soluble phenolase activity in the presence of 0.1 % SDS in leaf homogenate fractions is shown in Fig. 4. A membrane-free extract from isolated chloroplasts (C_s) and a membrane-free supernatant of a hypertonic leaf homogenate (H_s) have been used in this experiment. The stage of leaf development has not been determined. Compared to the control without SDS, 1 % SDS inactivates phenolase rapidly, 0.1 % SDS activates within the first 0.5–5 hr respectively, but strongly inactivates phenolase later on.

Thus in Fig. 3(d) (1 hr, 0.1 % SDS, 0°) activation of soluble phenolase is probably superimposed by inactivation in all stages of leaf age, but activation exceeds inactivation in young leaves and does not exceed, or is even zero, in senescing ones.

Although phenolase of young leaves can be activated *in vitro* to a level as high as to the activity in senescing leaves (Fig. 2), phenolase of senescing leaves can be activated by washing much more (Fig. 2(c)), even when using SDS additionally (Fig. 2(d)). Possibly SDS mediated inactivation of extracted phenolase in the washings may be responsible. Therefore SDS had been applied directly to membrane fractions P, omitting previous extraction of phenolase. Indeed from Fig. 3(e) it is evident, that under these conditions, rates of activation are as high in young leaves (3 weeks) as in senescing ones (5 weeks). Latent

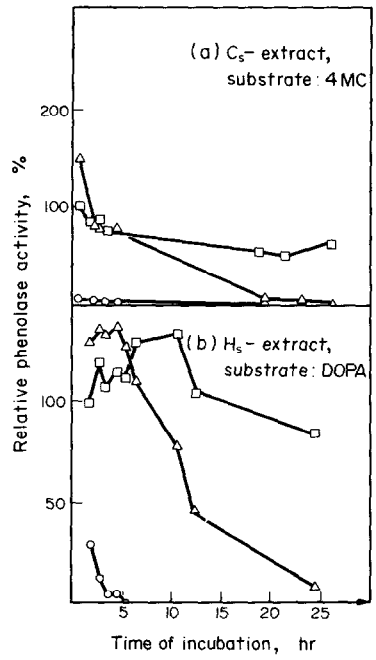

Fig. 4. Changes of activity of soluble phenolases during SDS-incubation. Samples were incubated at room temperature with SDS (final concn: ○——○ 1.0 %; △——△ 0.1 %; □——□ 0 % = control). SDS was added to (a) a colourless 30 000 *g* supernatant of hypotonically shocked isolated chloroplasts (C_s) containing less than 0.03 % of the homogenate chlorophyll, and to (b) a 30 000 *g* supernatant from a hypertonic leaf homogenate (H_s) containing less than 1.1 % of the homogenate chlorophyll. Chloroplasts were isolated from a hypertonic leaf homogenate according to [6]. Samples were frozen before incubation. Activities were estimated polarographically as described in Fig. 1 with 4-methylcatechol (4 MC) or DL-DOPA respectively. Activities of the control samples without SDS at 0 time were set to be 100 %.

phenolase from the membrane pellets can also be activated by acid shock at pH 3.5, by 4 M urea (according to [9]) and by alkali shock at pH 11.5. All of these procedures are less effective than SDS-incubation. Phenolase from supernatants is slightly inactivated under these conditions (data not shown).

Electrophoretic analysis

As has been published [1] phenolase forms, separable by electrophoresis, change during senescence and give rise to two forms, one "membrane form" and one "soluble form" (named X) which is responsible for the bulk of activity in senescing leaves. We have tried to determine whether phenolase in the repeated washings from leaves at different ages can be converted artificially *in vitro* by 4 M urea, acid shock, alkali shock 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 conversion be detected by electrophoresis.

Tracer experiments

Phenolase form X comprises most of the overall activity during, but not before senescence [1], and form X can be

Table 2. Incorporation of [^{14}C]amino acids into proteins of spinach primary leaves of different ages

	Leaf age (weeks)	3	5	7
a	Specific phenolase activity in the homogenate 70 min after homogenization per protein (μmol O_2/hr/mg)	2.79	4.76	3.63
b	Incorporation of ^{13}C into total leaf protein (dpm/mg)	218000 ± 600	114600 ± 400	95200 ± 700
c	Incorporation of ^{13}C into phenolase form X per unit of activity (dpm/unit†)	637 ± 12	28 ± 1	27 ± 1

† One unit catalyses the consumption of 1 μmol O_2/min.

Age of leaves and phenolase assay are as described in Fig. 1. For further details see Experimental.

isolated electrophoretically. Therefore this form was used for tracer experiments to elucidate whether it is synthesized at higher rates after the onset of senescence.

After incubation of leaf slices in ^{14}C-labelled amino-acid solutions, incorporation of radioactivity into total leaf protein and into form X was estimated (Table 2). Experiments were run with non-senescent 3-week-old primary leaves and with 5- and 7-week-old senescent ones. In this experiment 3-week-old leaves contained sufficient amounts of phenolase form X for analysis, but the main increase in activity occurred between 3 and 5 weeks of leaf age according to electrophoretic analysis. Even strongly senescent leaves incorporate considerable amounts of labelled amino acids into leaf protein (Table 2(b)). In contrast, phenolase form X is highly labelled in non-senescent leaves, but nearly not at all in senescing ones, although form X is increased during this time [1]. After onset of senescence ^{14}C-incorporation is reduced only by 50% into total leaf protein but is diminished by 95% into phenolase form X. Thus no *de novo* synthesis of phenolase during senescence is indicated.

DISCUSSION

It has been reported that in several plants synthesis of proteins and of some enzymes increases during senescence (e.g. [10]). This may apply to leaf phenolase as well. However another explanation for increasing phenolase activity during leaf senescence is enhanced liberation from thylakoid membranes [1, 6]. In olive fruits, for example, phenolase is shifted from a thylakoid bound to a soluble state during ripening, though overall activity declines in this case [18]. It is well known that several membrane bound enzymes are activated by changing the status of membrane lipids [11]. Thylakoid membranes are altered during senescence (e.g. [12]) and phenolase of spinach leaves [2, 6] and other plants (e.g. [5]) are membrane bound.

Arguments for the second explanation have been drawn up in another paper dealing with changes of phenolase activity and its multiple forms [1], but *de novo* synthesis could not have been ruled out. This paper states on the basis of tracer experiments that there is no net *de novo* synthesis of phenolase during senescence and gives evidence by experiments with activating agents that overall phenolase content of young leaves is sufficient to account for increase of activity in senescing leaves.

Experimental evidence is given that increase of phenolase activity during senescence is caused by facilitation of membrane unbinding of the enzyme. It has been shown earlier [6] and discussed in a foregoing paper [1] that *in vitro* activation in membrane containing fractions of leaf homogenates is due to release of latent phenolase from the thylakoid membranes but not to the washing out of an inhibitor. Membrane unbinding and activation of phenolase in buffered aqueous homogenate fractions is found during storage and chloroplast isolation procedures [6] which cause changes of thylakoid structure [17]. During onset of senescence, the structural integrity of thylakoids is lost [10, 12]. As shown by successive washings (Table 1) the stability of membrane association of phenolase decreases during senescence.

These findings support Weston's [13] assumption that increase of activity during storage of detached old tobacco leaves is due to activation of latent enzyme. Using inhibitors of protein synthesis, Weston has shown that young tobacco leaves, but not senescing ones, are capable of phenolase synthesis [13]. Additionally it must be concluded from this paper that the appearance of new multiple forms during senescence [1] is caused by interconversion rather than *de novo* synthesis.

There is one striking difference between activation during *in vitro* storage and increase in activity during senescence: there is no interconversion of forms during storage of homogenates (see also ref. [6]). An attempt to explain this difference has been made elsewhere [1] by assuming changes of membrane composition during senescence but not during *in vitro* ageing. The present paper gives support to this assumption: SDS which is able to solubilize membranes (and which ultimately denatures proteins) activates phenolase of membrane-containing fractions and even membrane-free extracts from non-senescent leaves only. Thus in young leaves there must be complexes containing latent phenolase which are sensitive to SDS, but not or only slightly sensitive to ageing by storage. Because of the SDS-mediated inactivation superimposing activation of phenolase, conversion of multiple forms by SDS cannot be studied by electrophoresis. However *in vitro* conversion of spinach phenolase from acetone dry powder [8], but not from crude extracts (this paper), allows the assumption that multiplicity of forms is due at least partially to different complexes of one enzyme derived from the thylakoid membrane.

EXPERIMENTAL

Primary leaves from field-grown spinach plants (*Spinacia oleracea* L. cv Matador) were harvested at different ages and hypotonic homogenates were prepared as described elsewhere [1]. Membrane-free supernatants (S) and resuspended membrane sediments (P) from a centrifugation (1 hr, 30000 *g*) were investigated for phenolase activity with a polarographic assay system [1]. Activation of latent phenolase was followed by measuring the activities after storage in an ice bath. Solubilization of bound phenolase was performed by repeated washing of 1 ml fractions of resuspended membranes with fresh 0.067 M Na, KPi: buffer, pH 6.8. Before centrifugation (1 hr, 30000 *g*) the fractions were frozen (−18°) and thawed twice. Activation with SDS was performed by adding the same vol. of 0.2% SDS soln in 0.067 M NaPi buffer pH 6.8 and storing for 1 hr at 0° before phenolase assay. Buffers used in expts with SDS were composed of Na salts only. Disc-electrophoretic analysis is

described elsewhere [1]. For the tracer expts, leaves of different ages were cut into 3 mm strips and infiltrated with a soln containing 5 μCi (= 1.85×10^5/sec) ^{14}C-labelled amino acids from protein hydrolysate (modified according to [14]). Leaves were incubated for 17 hr in a moist chamber at room temp., washed twice with H_2O and homogenized in 2 vol. of a hypotonic grinding medium [1] with a Virtis homogenizer (3×10 sec, 45000 rpm). The homogenates were stored for 30 min in an ice bath, centrifuged (1 hr, 30000 g) and the pellets were resuspended with fresh grinding medium and frozen to activate latent phenolase. The resuspended membranes were spun down and the combined supernatants were concd by ultrafiltration. The proteins were separated by two subsequent runs in a slab gel system according to [15], using 0.125 M Tris–borate buffer pH 8.9 for the first run and 0.2 M Tris–borate buffer pH 6.6 for the second. Phenolase form X was extracted from the gel with 0.067 M Na, KPi buffer pH 6.8. Incorporation of labelled amino acids into form X and into the 5% TCA-ppt. protein of the homogenate was detected by liquid scintillation counting with Bray's soln. Correction of cpm to dpm was performed by using an ext. standard.

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