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THE LATENCY OF SPINACH CHLOROPLAST PHENOLASE

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Key Word Index—*Spinacia oleraceae*; Chenopodiaceae; Spinach; phenolase; latent enzyme; low MW inhibitor; inactive enzyme complex.

Abstract—The latent phenolase in spinach chloroplast membranes could be activated by treatment with various detergents. Examination by thin-layer gel filtration showed the presence of two active proteins (one with lower MW called protein A and the other, protein B). The protein B was converted to A by dilution or on standing, and the latter conversely to the former by concentration. On freezing, an extract of the acetone powder of the chloroplasts, phenolase activity was strikingly reduced, and this is ascribed to an association of the protein A and a low MW (diffusible) substance giving rise to an inactive enzyme-inhibitor complex. The activity declined from autumn to winter, and it appears that the second type of latency due to the formation of the above complex is also involved.

INTRODUCTION

Phenolase (*o*-diphenol : O_2 oxidoreductase) has been known to be widely distributed among plants, and its location in subcellular particles is now confirmed from the investigations by sucrose density gradient centrifugation [1, 2]. In the chloroplasts of some plants, this enzyme is bound to the membranes and can be extracted by detergents [3–9] and proteolytic enzymes [10]. The extraction of membrane phenolase was often accompanied by its activation [3, 10], so that the enzyme has been regarded as being latent [4, 10]. The latency of this enzyme has been reported in the chloroplasts of sugar beet [10, 11], spinach [2, 4, 12] and grape [13, 14].

While we studied some aspects of the metabolism of phenolic compounds using isolated chloroplasts [7, 15–17], it has been found that a low MW substance is partly responsible for the latency of spinach chloroplast phenolase.

RESULTS

Extraction and activation of enzyme

The isolated chloroplasts of spinach leaves showed only a slight phenolase activity, which could be enhanced 4–9 fold with detergents. The enzyme was not dissociated merely by standing in the buffer, whilst a significant activity could be found in the 25000 *g* supernatant fractions of the detergent-treated digests, suggesting that the detergent effected both the activation of latent enzyme and its dissociation (release) from the membrane structure. The dissociation rate however varied with different detergents, and this means that the activation of the enzyme was not necessarily accompanied by its complete dissociation (e.g. in the case of Triton X-100), that is, a part of the activated enzyme was still bound to the ruptured membranes (Table 1).

The existence of two enzyme forms and their interconversion

To determine whether the enzyme is in multiple forms as found in the chloroplasts of other plants [5, 6], the supernatant of the chloroplast brei was examined by thin-layer gel filtration (TLG) on Sephadex G-150 layers. Two protein species with this activity were detected, which seem to be derived mainly from the stroma. The major phenolase with higher activity and lower MW is designated protein A and the minor prenolase with lower

Table 1. Activation and dissociation of spinach chloroplast phenolase with various detergents (October chloroplasts)

Detergent	Activity* of digest† (a)	Activity of supernatant‡ (b)	Dissociation rate (b)/(a) × 100
Na-dodecylsulphate	47·0	46·1	98
Na-deoxycholate	39·3	39·4	100
Triton X-100	19·5	6·8	35
Tween 80	21·4	7·2	34
Digitonin	20·0	14·2	71
None (control)§	5·2	trace	negligible

* μl O_2/min/sample equivalent to 1 mg of chlorophyll, with $6{\cdot}7 \times 10^{-3}$ M catechol. † The chloroplast suspension after standing for 1 hr in 1% detergent dissolved in 10 mM Pi buffer pH 6·8. ‡ Supernatant of the digest at 25000 *g* for 30 min. § The chloroplast suspension stood for 1 hr in 10 mM Pi buffer pH 6·8.

Table 2. Purification of the protein species A and B

Fraction		Days after extraction	Protein (mg)	Total activity*	Specific activity†	"Purification"
I	Original extract	0	146	10·4	7·18	1·0
II	40–60% $(NH_4)_2SO_4$ precipitate	0	50	7·99	16·0	2·2
	" "	9	50	53·2	106	14·8
III	Sephadex G-200 eluate					
A	Fraction No 24-28	10	10·4	24·2	233	32·6
B	Fraction No 17-19	10	3·7	7·27	196	27·3

* Unit × 10^{-2}. † Unit/mg protein.

activity and higher MW designated protein B. The treatment with Triton X-100 and trypsin [2] gave a similar pattern, but stronger coloration by both proteins were observed as compared with that by non-treated chloroplasts, due to the liberation from the membrane (the protein molecules were apt to disperse in the gel in the presence of Triton X-100).

Partial purification of the two proteins was then attempted (Table 2). TLG examination for proteins with phenolase activity showed that interconversions between native proteins and their conversion into artefact protein (s) (designated as 0-A) could readily occur in the course of purification. Fraction II contained a large amount of 0-A, which could be removed by standing for about 10 days, and this was accompanied by an increase of the activity (it appeared that 0-A was converted to A, but no direct evidence was obtained). An elution profile (Fig. 1, upper), which coincided well with the TLG pattern with regard to the activity of two enzymes, was obtained when the 40–60% $(NH_4)_2SO_4$ precipitate from 100 mg of acetone powder was dissolved in 5 ml of buffer followed by fractionation on Sephadex column. Fraction III B contained the protein B as well as A. A two dimensional TLG of this fraction showed that B was partly converted to A even when the second run was done immediately after the first run. The conversion was assumed to be due to a dilution of B on the gel. This was verified by diluting the above fraction. Storage for a long time also caused this conversion: a complete disappearence of B and an increment of A were observed after standing for 2 weeks at 0°. Contrarily to this, the concentration of protein solutions resulted in the conversion of A into B. When the precipitate from 100 mg of powder was dissolved in less than 1·25 ml of buffer, an increased peak of B and decreased of A were seen in the elution profile (Fig. 1, lower). Concentration of Fraction III A with Sephadex G-25 (coarse) brought about a distinct appearence of B.

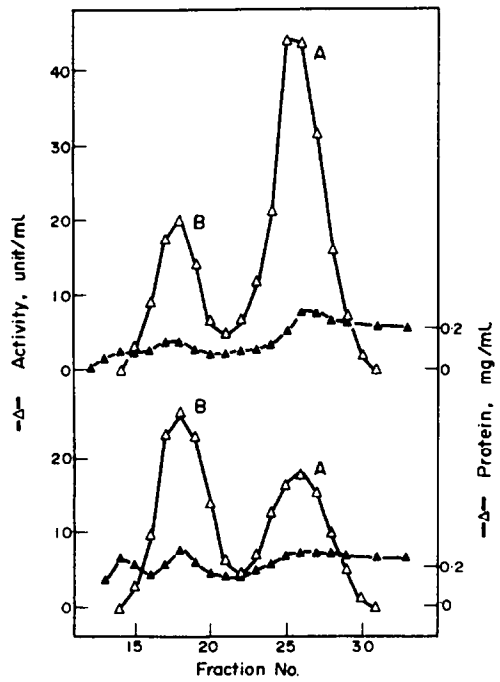

Fig. 1. Elution profiles of Fraction II on Sephadex G-200 column. Upper graph; the precipitate from 100 mg of the powder by 20–40% $(NH_4)_2SO_4$ precipitation was dissolved in 5 ml of buffer, lower; in 1·25 ml.

Inactivation on freezing and effect of dialysis

When the Fraction I (abbreviated as F-I) was stored in the frozen state, it was found that the phenolase acti-

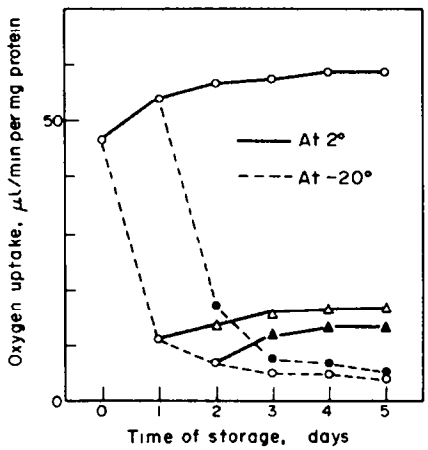

Fig. 2. Reduction of phenolase activity of Fraction I on freezing. —○—; stood at 2° throughout, --○--; stood at −20° throughout and --●--; stood at 2° for 1 day then frozen. —△— and —▲—; frozen 1 and 2 days, respectively, then returned to liquid state.

Table 3. Effect of dialysis on the activity before and after freezing

	Activity*	Relative activity
Fraction I	58·8	100
Dialysed	68·1	116
Followed by freezing	57·1	97
Frozen	14·1	24
Followed by dialysis	11·6	20

* μl O_2/min/mg protein.

vity strikingly decreased as much as 80%. No decrease in activity occurred in F-I which was held unfrozen at 2° and activity rather tended to increase. The once-frozen F-I did not recover its original level of activity, although a slight increase was observed (Fig. 2). Such a reduction was however not brought about when F-I was subjected to dialysis before freezing, but no recovery was achieved for the once-frozen F-I by dialysis (Table 3).

Similarily, the protein fraction obtained by $(NH_4)_2SO_4$ precipitation and gel filtration, being free from low MW substances, showed no significant loss of activity on subsequent freezing. The activity in an acetone precipitate was reduced by freezing, but this decrease was reduced by prior dialysis before freezing (Table 4). All these facts suggested that F-I contained a low MW substance (designated as X) which, on freezing, exhibited an inhibitory effect on the enzyme in active form. Its presence was supported by an experiment where the dialysate of F-I was mixed with its diffusate followed by freezing. A distinct reduction of the activity was confirmed (Table 5).

TLG examination for the frozen F-I showed that protein A, but not protein B, was remarkably reduced, so that freezing seemed to result in the formation of an inactive complex from protein A and X (this would be designated as A-X). The freezing effect could not be observed in the purified protein fraction of A (Fraction III A) for 2–3 days after its elution from a Sephadex column, but a significant reduction of activity was brought about by freezing the same fraction after standing several days (Fig. 3).

Characterisation of the inactive complex

The result in Fig. 3 can best be accounted for by postulating that Fraction III A contained free protein A as well as inactive protein complex A.X (the MW of the 2 protein species is of similar order) and the complex partly dissociated to give rise to free X, which in turn formed the complex with pre-existing or dissociated A on freezing (that standing at 0° resulted first in an increase, then in a decrease of activity was probably due to the association of the complex and inactivation of A, respectively).

Using Fraction I, we then attempted to dissociate A and X from the complex. If the dissociation would occur under mild conditions other than standing, then the

Table 4. Effect of freezing on the activity of various protein fractions

	Activity* Original (a)	After freezing (b)	(a–b)/a × 100
Fraction I	55·0	11·0	80
$(NH_4)_2SO_4$ precipitate (100%)	63·6	63·3	0
Acetone precipitate (3 vol)	60·6	33·3	45
Acetone precipitate dialysed	70·0	63·1	10
Sephadex G-25 eluate	38·9	36·3	7

* μl O_2/min mg protein.

Table 5. Effect of diffusate on the activity

Component	Original (a)	Activity* After standing overnight (b)		(a-b)/a × 100
Dialysate + Diffusate	112	2°	119	−6
		−20°	71	37
Control (dialysate + 0·05 M buffer)	108	2°	107	1
		−20°	99	8

*μl O_2/min/mg protein. Experimental procedure; see Experimental.

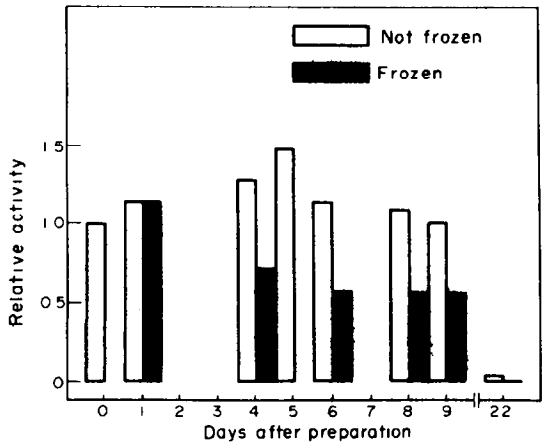

Fig. 3. An increasing freezing effect in the Fraction III A stood at 0° after its preparation from the Sephadex column. A part of this Fraction had been frozen overnight before estimating the activity.

phenolase activity should increase. Treatment with acid-alkali [20, 21] and 6 M urea [21] was ineffective. Heating at 50° for 5 min resulted in a complete loss of activity and Triton X-100 (1%) was rather inhibitory. Sonication brought about a clear activation, but did not give reproducible results. We therefore tried to liberate X under conditions which lead to the inactivation of the enzyme. An exhaustive dialysate of F-I, being devoid of freezing effect, was made alkaline, heated and, after acidification, low MW substances were extracted with ethyl acetate. The mixture of the ethyl acetate extract and the Fraction III A reduced the activity on freezing, whilst no reduction or differences could be found on TLG in the three controls (the above mixture at 0°, and mixtures at 0° or −20° with an equal volume of water instead of the ethyl acetate extract).

Deterioration of enzyme activity with time of harvest

The phenolase activity in the Triton X-100 digest which was treated for 1 hr, was relatively high until early October, but it gradually decreased and almost completely disappeared during middle November (Fig. 4). A suspicion that the above decrease might merely be due to the turnover of the enzyme itself was ruled out by the fact that, even in late November, by which time the activity in 1 hr-treated digests could hardly be observed, it could be increased by longer treatment (4 days or more) to a level comparable to that of 1 hr-treated digest in early October.

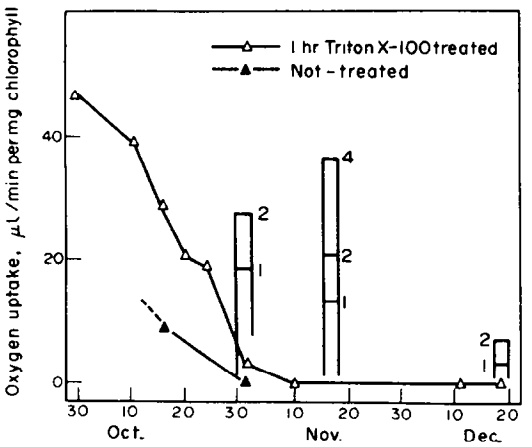

Fig. 4. The seasonal change of the phenolase activity of the non-treated and 1 hr-treated Triton X-100 digests of the chloroplasts, and the effect of longer treatment on the activity in late season. The figures on the right side of columns represent the length of treatment in days.

As shown in Fig. 2, standing of F-I (this fraction was prepared from chloroplast digest treated with Triton X-100 for 1 day) resulted in an increase of its phenolase activity. This was only the case for F-I from the chloroplasts harvested before November. F-I from the December chloroplasts showed neither activity nor its enhancement on standing. It seems however that protein A is not completely turned over, but exists in an inactive (latent) form as the A-X complex, because the protein A-reducing activity of the ethyl acetate extract was confirmed also in F-1 from the December chloroplasts.

DISCUSSION

The results presented show that the latency of spinach chloroplast phenolase is achieved in two ways. One is by a firm binding of the enzymes with the membrane structure of the chloroplasts. The second is by formation of an inactive complex from active protein and a low MW substance. The latency is therefore controlled, so far as spinach phenolase is concerned, by two different mechanisms which should be distinguished from each other: The former is an event at subcellular level, whilst the latter at molecular level.

The former case has been reported in the catechol oxidase (phenolase) of sugar beet chloroplasts, which is activated by proteolytic enzymes through cleavage of its peptide bond attached to the membrane [10, 18]. On the other hand, activation of latent phenolase at a molecular level has been shown in several plants. In broad bean, a dissociation of a phenolase-inhibitor complex or a configurational change in prophenolase [19], or a partial denaturation as well as a rearrangement of the tertiary structure of the proteins [20, 21] have been postulated to be involved in the activation. The activation of grape phenolase has been ascribed to conformational change in the enzyme accompanied primarily by an increase in V_{max} for oxygen [13].

Standing of F-I from the chloroplasts before November resulted generally in an enhancement of activity, whilst this was not the case for F-I from December chloroplasts. It might be reasonable to postulate that there are several types of A.K complex varying in their "tightness" of association between A and X. The association in one type is more loose so that the complex can partly dissociate to its components, and in the chloroplasts before November the complex of this type seems to exist in an appreciable amount. In another complex, the association is tighter and will not dissociate under mild conditions, and it seems that in the chloroplasts in December the complex of the latter type predominates. Later in the season, therefore, an unclarified mechanism, substituting for *in vitro* freezing procedure, should operate probably in the membrane part of the chloroplasts.

The present studies did not concern the nature of association brought about by freezing. Attempts to lower the phenolase activity of F-I by other procedures than freez-

ing were unsuccessful, nor was any reproducible procedure found for converting the inactive enzyme to the active form.

EXPERIMENTAL

Spinach (*Spinacia oleracea*) used in the present experiments were all obtained in markets from September–December.

Preparation of the chloroplasts. Leaves, washed in H_2O and detached from petioles, were blended in 5 vol of a cold medium containing 0·4 M sucrose, 0·05 M Tris–HCl buffer pH 7·8 and 10 mM NaCl. The homogenate was filtered through silk, and the filtrate centrifuged at 150 *g* for 2 min. The ppt was discarded and the upper suspension further subjected to a centrifugation at 3000 *g* for 5 min. The pellet was carefully washed with 0·4 M sucrose.

Preparation of acetone powder. The chloroplast pellet thus obtained was suspended in H_2O (pellet from 1 kg leaves in 100 ml) and an equal vol of 2% Triton X-100 in 20 mM Pi buffer at 6·8 was added. The suspension was stirred for 1 hr and further stood overnight at 2°. The digest was centrifuged at 25000*g* for 30 min, and 3·5 vol of cold Me_2CO at −20° were added to the supernatant. The ppt. was collected by centrifugation and repeatedly washed with Me_2CO until no colour was observed. The ppt was dried in a desiccator and the powder stored at −20° until used (2·8 g from 1 kg leaves). The loss of the activity was less than 10% by 1 yr storage at −20°.

Preparation of the Fraction I (F-I). The powder was suspended in 100 vol of 10 mM Pi buffer pH 6·8 and the suspension stirred for 1 hr. It was centrifuged at 25000 *g* for 30 min, and faint brownish supernatant was used as Fraction I.

Partial purification of the enzymes. To the Fraction I prepared as above, saturated $(NH_4)_2SO_4$ was added to 40% saturation and after 1 hr standing the ppt was discarded. The supernatant was brought to 60% saturation, the proteins collected by centrifugation and dissolved in 10 mM buffer (ppt from 100 mg of powder in 5 ml). The soln (Fraction II) was applied on Sephadex G-200 column (2·5 × 30 cm) and 5 ml fractions collected with the same buffer by upward flow to give rise to Fraction III A and B.

Thin-layer gel filtration (TLG). Samples were applied to a TLG plate of Sephadex G-150 (superfine; thickness; 1 mm) and developed with 10 mM buffer for 4–5 hr at room temp. The enzymes were located from a red colour which appeared within 30 sec after spraying a solution containing catechol and sulfanilic acid.

Dialysis and standing. Dialysis was carried out at 2° overnight using Visking cellophane tubing (size; 8/32) against 200 vol of 10 mM Pi buffer pH 6·8. Standing of enzyme solns was carried out at 0°.

Freezing and thawing. Freezing was for 18 hr at −20°. The frozen sample was thawed at about 10° within 30 min.

The estimation of phenolase activity. An ordinary manometric method was employed. In the flask were placed 0·5 ml of enzyme soln (chloroplast suspension or detergent treated digest) and 0·5 ml of 0·2 M Pi buffer pH 6·8, and in the side arm 0·5 ml of 10 mM catechol soln. The whole flask was preincubated for 10 min before the reaction was run at 30°. In some expts, the colorimetric method of Shiroya [22] was used with some modifications. A mixture consisting of 0·5 ml of enzyme soln, 0·5 ml of 10 mM catechol, 1 ml of 10 mM sulphanilic acid and 2 ml of 0·2 M Pi buffer pH 6·8 was incubated at 25°. One enzyme unit is defined as the amount of enzyme which increases the A at 500 nm by 0·01 in 1 min under the specified condition.

The estimation of chlorophyll and protein. Chlorophyll and protein were estimated according to refs [23] and [24].

Extraction of substance x. The original extract from Me_2CO powder prepared at various dates was made to pH 11 with 0·1 M NaOH and the soln was heated for 10 min at 95°. After acidifying to pH 3 with N HCl, the substance was extracted with an equal vol of EtOAc 5×, and the combined extract was replaced by H_2O. Because of a contamination of other low MW substances (e.g. substance of inhibitory nature) and of the volatile nature of the substance, a well-defined quantitative estimation of x could not be made in this experiment.

Mixing experiment of the dialysate and diffusate. Fraction I (10 ml) was dialysed overnight against 20 ml of 10 mM Pi buffer pH 6·8. The protein in this dialysate was precipitated by satd $(NH_4)_2SO_4$, dissolved in 5 ml of the same buffer and the soln dialysed to remove salt (final vol 5·5 ml). 10 ml of the diffusate of the first dialysis was lyophilized, the residue taken in 4 ml of H_2O, and the soln used as the diffusate. 0·8 ml of the second dialysate was mixed with 3·1 ml of the diffusate followed by standing at 2° or −20°. Controls were run, which consisted of the second dialysate and pi buffer of the same concn as that of the dialysate-diffusate system.

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