Phytochemistry, 1978, Vol 17, pp 1427–1429 © Pergamon Press Ltd Printed in England 0031-9422/78/0801-1427 $02.00/0

SHORT REPORTS

ACTIVATION OF LATENT PHENOLASE FROM SPINACH CHLOROPLASTS BY AGEING AND BY FROST

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(*Received* 13 *January* 1978)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach polyphenoloxidase; latency; activation; ageing; frost; chloroplasts.

Abstract—Activation of latent phenolase by freezing and thawing occurs in both thylakoid sediments and membrane washings from spinach chloroplasts, while ageing and digitonin treatment activates membrane-bound enzyme only. Disc electrophoresis reveals that frost converts a soluble, latent phenolase to an active form after its release from the thylakoid membrane. Ageing of membranes containing latent phenolase results in direct liberation of other active forms. There are further active, soluble forms, which are exclusively found in the chloroplast stroma fraction.

INTRODUCTION

Phenolase (polyphenoloxidase) activity has been shown to be tightly bound to thylakoid membranes of several plants, as in spinach [1–4] but the enzyme can be separated by simply washing the membrane preparation [5, 6]. We have found that latency of chloroplast phenolase and its activation is directly related to association and dissociation of the membrane-bound enzyme. While this kind of activation, mediated for example by detergents, ageing and washing, is caused by release of enzyme from the thylakoid membrane [7], freezing and thawing cause another kind of activation. These different activations and resulting electrophoretic patterns of phenolase forms are reported in this paper.

RESULTS AND DISCUSSION

Polyacrylamide disc electrophoresis of fractions from isolated chloroplasts (Fig. 1) as well as slab gel electrophoresis reveal several phenolase forms which separate into two groups: (1) A fast-moving group of soluble forms (VII–X) predominating in older leaves, but present as well in young leaves, remaining completely within the supernatant after hypotonic treatment of intact chloroplasts. (2) A group of membrane-associated forms (IV–VI) which can be consecutively extracted from the membrane sediment by repeated washing with aqueous media [6, 7]. Storage as well as digitonin treatment of membrane-containing fractions enhances phenolase activity considerably, whereas little or no activation takes place in membrane-free fractions under the same conditions (Table 1). From fractions containing isolated thylakoid membranes all apparent activity can be extracted by washing, but about the same amount of activity is restored in the resuspended membrane sediment and can be separated by further washing from the membranes. This can be repeated several times [6].

In contrast to digitonin or storage activation in membrane fractions, freezing and thawing causes activity enhancement not only in membrane-containing fractions but in membrane-free supernatants as well (Table 1).

Gel electrophoresis of fractions activated by these different treatments reveal typical patterns of enzyme forms and browning intensity after DOPA incubation of the gels. Ageing increases forms IV–VI considerably in membrane-containing fractions. These forms have been released from membranes during storage (Fig. 1b, c), because there is no change of phenolase patterns in membrane-free fractions during ageing and because only

Table 1. Influence of digitonin and/or frost on phenolase activity of membrane-containing and membrane-free chloroplast fractions

Sample	Phenolase activities				
	without treatment	30 hr aged at 0–3°	digitonin treatment	frost treatment	digitonin and frost treatment
(a) Isolated chloroplasts (hypotonic)	0.16	1.13	10.42	4.57	57.05
(b) Stroma (30000 *g*-supernatant from (a))	0.49	0.595	0.43	2.08	2.08
(c) membranes (30000 *g*-sediment from (a))	0.30	3.62	—	—	—
(d) washed membranes	0.946	1.91	11.24	12.79	33.90

Isolated chloroplasts (1000 *g*-sediment) were suspended in hypotonic shock medium (a). After high dilution of (a) with the hypotonic medium, the stroma containing supernatant (b) was separated from membranes by centrifugation at 30000 g. Membranes were suspended in the hypotonic medium (c). Precipitation and resuspension of (c) were repeated × 2 to get washed membranes (d). All treatments were done in the dark. Digitonin was used in a final concn of 0.5%. Frost means six days storage at −18° and thawing at 20°. The phenolase activity (μM O_2/mg chlorophyll × hr) was estimated polarographically.

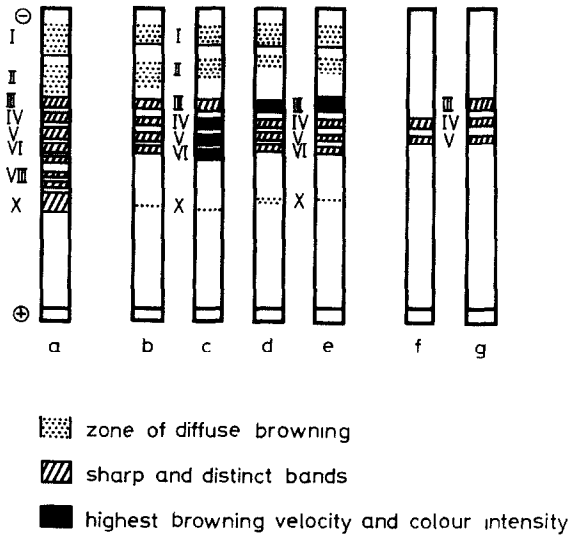

Fig. 1. Disc gel electrophoresis of spinach chloroplast phenolase Scheme representing the distribution pattern and colour intensities of bands after DOPA incubation in gels of several chloroplast fractions isolated from young leaves. (a) All detectable phenolase forms summarized in one diagram. (b–e) Stroma containing chloroplast extracts. (b) Isolated chloroplasts were subjected to shock (1 hr, 3°). Membranes were removed by centrifugation (30000 *g*). 1460 μg protein/column of the supernatant. (c) The same as (b), but the shocked chloroplasts were stored 72 hr at 3° in the dark before centrifugation. 720 μg protein/column. (d) The same as (b), but the shocked chloroplasts were stored 72 hr at −18° and thawed at 20° before centrifugation. 720 μg protein/column. (e) The same as (d), but the membranes were removed by centrifugation before storage at −18°. 1460 μg protein/column. (f–g) Extracts of washed chloroplast membranes. (f) 30000 *g* membrane sediment from (b) twice resuspended in buffer and pptd by centrifugation. 800 μg protein/column of the second supernatant. (g) The same as (f), but the second supernatant stored 24 hr at −18° and thawed at 20°. 800 μg protein/column.

forms IV–VI are released from washed membrane sediments (Fig. 1f).

Frost treatment, in contrast, causes significant intensification of form III only. This form is absent or rarely found as a weak zone in 0° preparations of fresh, aged or even detergent-treated chloroplasts (Fig. 1g). There is no change in distribution or browning of the other phenolase forms, suggesting that form III is generated from an inactive precursor extracted from thylakoid membranes. Digitonin treatment does not change distribution patterns or browning intensities of electrophoretically separated phenolase forms. Neither digitonin or acetone extraction nor storage has any influence on formation of zone III.

In earlier publications, it has been concluded that activation of membrane-bound phenolase is brought about by alteration of the chloroplast lamellar system [1–3]. We have found this activation of latent spinach phenolase to be the result of its dissociation from the thylakoid membrane [6, 7]. In contrast, frost activation, as reported here, of a soluble latent phenolase is different. Both types of activation produce special electrophoretically separable enzyme forms in spinach chloroplast fractions.

For the first type of activation it is not known whether the enzyme becomes active immediately as a result of its removal from associating membrane constituents or if it is activated shortly after. However, liberation from the membrane is correlated with its activation. The other mode of activation which results in the formation of form III is different, because of its electrophoretic peculiarity and the need for frost. Although the latent precursor is derived from the membranes as well, it is stable in the aqueous washings after liberation.

This frost activation contrasts with the results of Sâto and Hasegawa [4] and Sâto [8] who found inactivation of extracted spinach phenolase following freezing and thawing, apparently brought about by binding to a low molecular weight inhibitor which is assumed to be a volatile acid. The experimental conditions are, however, different: we used chloroplast fractions in aqueous media, whereas Sâto used acetone powder from chloroplasts. In our hands, acetone treatment of soluble chloroplast phenolase caused 60–70 % of inactivation. Thus acetone may alter the state and activity of the enzyme, more drastically than the ageing and freezing reported here. Although we do not know the molecular mechanism of frost activation, it seems unlikely that form III is a result of frost-dependent modification [9] of another phenolase form since there were no changes in the disc electrophoretic pattern after freezing and thawing. It is more likely that conformational changes of prophenolase [10, 11] or dissociation of inhibitor complex are involved. Madhosingh [12] reported two low molecular weight mushroom inhibitors of DOPA oxidation and Harel *et al.* [13] have shown inhibition of mushroom tyrosinase by a low molecular weight peptide inhibitor obtained from *Dactylium dendroides*.

EXPERIMENTAL

Chloroplasts were prepared from primary leaves of young spinach (*Spinacia oleracea* L.) plants grown in a growth chamber under a 10 hr day at 18°. The plants received light from Osram HQIL-lamps 400 W (distance 1.6 m). Chloroplast preparations were obtained by differential centrifugation. The grinding medium contained 0.6 M sucrose in 0.01 Na phosphate/HCl buffer pH 7.8 Hypotonic shock was produced by suspending chloroplast sediments (1000 *g*) in 0.002 M Tricin/NaOH buffer pH 7.8 containing 0.035 M NaCl and 0.001 M $MgCl_2$. Thylakoid membranes were sedimented at about 30000 *g* for 1 hr. Supernatants did not contain more than 0.05 μg of chlorophyll per ml. Phenolase activity was estimated polarographically at 25° with an YSI oxygen electrode 5331. The air-saturated reaction mixture contained 4-methylcatechol in a final concn of 7.5 mM in a total vol. of 3 ml (0.067 M phosphate buffer pH 6.8 according to Sörensen). Further, a photometric assay [14] was used, as described elsewhere [6, 7]. The kinetics of absorbancy decrease at 265 nm due to oxidation of ascorbic acid by enzymatically oxidised 4-methylcatechol was measured for at least 10 min. Protein assay were as ref. [15], chlorophyll was estimated using the McKinney [16] method. Digitonin was recrystallized twice from EtOH and used according to ref. [17]. Polyacrylamide gel electrophoresis was performed applying disc electrophoresis according to ref. [18] (system No. 1a) and slab gel electrophoresis according to ref. [19]. *Ca* 700 to 1500 μg of soluble protein could be applied to each disc or slab gel electrophoresis without overloading. Enzyme activity in the gels was assayed by incubating the gels in oxygen-satd phosphate buffer (Sörensen) pH 6 containing a final concn of 5 mM DOPA. The colour developed after 30 sec to 4 hr.

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Phytochemistry, 1978, Vol 17, pp 1429–1431 ©Pergamon Press Ltd Printed in England 0031-9422/78/0801-1429 $02 00/0

PROPERTIES OF POTATO α-GLUCOSIDASE

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; α-glucosidase; properties

Abstract—Potato tuber α-glucosidase has an isoelectric point of 4.7 and an apparent MW of 120000. The enzyme has a neutral pH optimum (pH 6.5–7.0) and a K_m of 0.21 mM for *p*-nitrophenol-α-D-glucoside at pH 6.8 and 30°. Maltose and higher maltosaccharides are also substrates. The enzyme exhibits transglucosidase activity.

INTRODUCTION

α-Glucosidases (α-D-glucoside glucohydrolase, EC 3.2.1.20) are exoenzymes which catalyse the hydrolysis of glucose units from the non-reducing ends of α-linked glucans. The enzymic activity has been demonstrated in a number of plant extracts [1–10] and was first detected in potato tuber extracts by Hutson and Manners [1] but the enzyme was not characterised in any detail. We report here some of the properties of a partially purified preparation of the potato tuber enzyme.

RESULTS AND DISCUSSION

The partially purified α-glucosidase preparation was found to apparently contain only one activity which hydrolysed *p*-nitrophenyl-α-D-glucoside. One activity band of low electrophoretic mobility was detected on polyacrylamide gels. On isoelectric focusing in the pH range pH 3.0–10.0 a single activity peak, having an apparent isoelectric point of 4.7, was obtained. When subjected to gel filtration on a Sephadex G-200 column, the α-glucosidase activity was eluted as a single symmetrical peak having an apparent MW of 120000.

The effect of pH on the stability and also on the catalytic activity was tested in the pH range 2.5–9·0 in McIlvane buffers. The α-glucosidase activity was stable in the pH range pH 6.0–9.0 when stored at 4° for 16 hr, and was stored in 0.1 M potassium phosphate buffer, pH 6.8, for 6 months at −20°, with no loss in activity. Maximal activity on *p*-nitrophenyl-α-D-glucoside was observed in the pH region 6.5–7.0. Little activity (0–15% of maximal activity) was observed in the pH region pH 2.5–4.5, indicating the absence of acid α-glucosidase activity such as have been observed in other plant tissues [1–10].

Using *p*-nitrophenyl-α-D-glucoside at concentrations varying from 0.25–2.0 mM and standard assay conditions (pH 6.8, 30°), a Lineweaver–Burk plot was obtained. The value of K_m, found from the plot, was 0.21 mM and this value is similar to that reported for the yeast enzyme [11]. At 5.0 mM and higher concentrations of *p*-nitrophenol-α-D-glucoside substrate inhibition was observed. EDTA (10 mM) and 2-mercaptoethanol (10 mM) had no effect on the enzymic activity. The α-glucosidase activity was inhibited (100%) by Tris (0.1 M) at pH 6.8. Inhibition of other α-glucosidase preparations by Tris has been extensively documented [9, 12–16].