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PHENYLALANINE AMMONIA-LYASE ACTIVITY OF PROTOPLASTS: *IN VITRO* INHIBITION BY MANNITOL AND SORBITOL

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco leaf; protoplasts; phenylalanine ammonia-lyase; *in vitro* inhibition; mannitol; sorbitol.

Abstract—Phenylalanine ammonia-lyase (PAL) activity was strongly inhibited *in vitro* by D-mannitol and D-sorbitol at concentrations exceeding 50 mM, inhibition being complete at 150 mM. These two sugars are the most frequently used plasmolytica (at concentrations from 0.4 M to 0.8 M) during isolation of mesophyll cells or of protoplasts. D-Glucose, D-mannose and D-sorbose were also inhibitory, but at higher concentrations. In contrast, sucrose did not inhibit PAL activity. The degree of inhibition by mannitol and sorbitol was lowered by increasing the concentration of the substrate, L-phenylalanine. Inhibition was reversible and enzymatic activity was fully recovered when crude extracts contaminated with these sugars were diluted. If protoplasts are to be assayed for PAL activity, the classical procedures for their isolation should be modified in order to completely remove mannitol or sorbitol, or a differential dilution procedure (described) should be used.

INTRODUCTION

We have already [1] reported that phenylalanine ammonia-lyase (PAL) activity is a good biochemical marker of the necrotic reaction of the leaves of hypersensitive tobacco plants infected by tobacco mosaic virus (TMV). Furthermore, Otsuki *et al.* [2] have shown that the limitation of virus multiplication that is found in leaf cells surrounding necrotic lesions is not detectable in protoplasts or mesophyll cells isolated from the same hypersensitive varieties and infected thereafter by TMV. These results and the absence of any visible symptoms of necrosis suggested that this plant material behaved as a systemic host despite the presence of the N gene, which determines the necrotic response to TMV. To see whether the necrotic reaction was functioning in protoplasts inoculated with TMV or isolated from preinfected hypersensitive leaves, we tentatively used PAL activity as a biochemical indicator.

Current methods for isolating tobacco mesophyll protoplasts are based on the two-step procedure of Takebe *et al.* [3]: dissociation of the exposed mesophyll into single cells using pectinase, and digestion of walls of the isolated mesophyll cells by cellulase. Both steps are carried out in a hypertonic non-ionic medium, usually containing mannitol at concentrations from 0.6 to 0.8 M. When we first assayed isolated mesophyll cells or protoplasts, either healthy or TMV-infected, we surprisingly could not detect any significant PAL activity. Furthermore, only slight enzyme activity was measurable even after partially purified PAL had been added to these crude extracts. We found that this inhibition of PAL *in vitro* was caused by residual mannitol in the crude extracts.

The present paper describes the inhibition of PAL activity by mannitol and by some other compounds currently used to make hypertonic media. We also indicate several ways of isolating leaf cells or protoplasts for PAL assay. A method for measuring PAL activity even in the presence of residual mannitol or sorbitol is also described.

RESULTS

Effect of various sugars on PAL activity in vitro

Table 1 shows that D-mannitol and D-sorbitol strongly inhibited PAL activity while sucrose had no significant effect. D-mannose and D-glucose were also inhibitors, but

Table 1. Effect of various sugars on PAL *in vitro*

Sugar	Concentration (mM)	Inhibition (%)
D-mannitol	60	38
	100	83
	200	100
D-sorbitol	60	36
	100	86
	200	100
sucrose	100	0
	200	0
	400	0
	700	7
D-mannose	100	19
	200	49
D-glucose	100	0
	200	41
	400	53

PAL used for the inhibition tests was extracted from tobacco leaves and purified 50-fold by the procedure of ref. [4]. The enzyme was incubated in 0.1 M borate buffer, pH 8.8, with 0.1 mM L-phenylalanine and the various sugars at the concentrations indicated. Enzymatic activity was determined radiochemically [1].

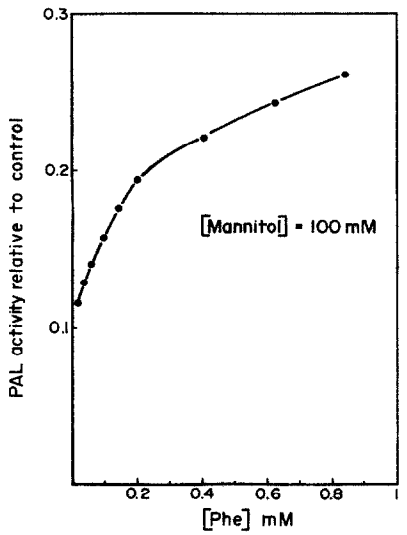

Fig. 1. Influence of the concentration of phenylalanine on the *in vitro* inhibition of PAL by mannitol. PAL was extracted from tobacco leaves and purified 50-fold by the method of ref. [4]. Two sets of samples were assayed in parallel with various concentrations of phenylalanine: samples of the first set were free of mannitol (controls), the samples of the second set all contained 100 mM mannitol. Enzymatic activity was determined radiochemically [1].

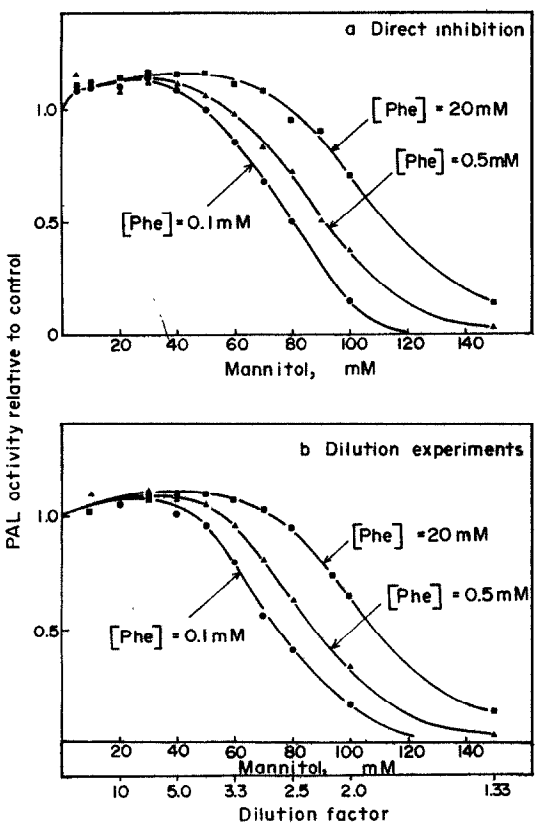

Fig. 2. Reversibility of the *in vitro* inhibition of PAL by mannitol. (a) A crude extract (0.1 M borate buffer, pH 8.8) of tobacco leaves was assayed for PAL activity in the presence of increasing concentrations of mannitol and of three different concentrations of phenylalanine. Enzyme activity is expressed relative to that of the control (the same crude extract free of mannitol). (b) To an aliquot of the crude extract in (a) was added borate buffer either alone (controls) or containing mannitol (final concentration 200 mM). Ten different dilutions of these two extracts were then assayed in parallel for PAL activity in the presence of the three concentrations of phenylalanine. Enzymatic activities are expressed relative to controls (identical dilutions of the crude extract free of mannitol).

Enzymatic activities were measured radiochemically in the case of incubations with 0.1 mM phenylalanine and using Zucker's spectrophotometric method [12] in the case of incubations with 0.5 mM and 20 mM phenylalanine.

at higher concentrations. The two former sugars are those most frequently used to provide the osmotic pressure required for the isolation of protoplasts using pectinase and cellulase sequentially [2] or simultaneously [5–7], and in culture media for plant protoplasts [8]. When suspensions of freshly prepared or cultured protoplasts are ground in the presence of buffer, the crude extracts obtained are contaminated by residual hypertonic medium and therefore contain these sugars at concentrations that more or less inhibit PAL activity. If, for instance, a given volume of protoplasts suspended in 0.7 M mannitol or sorbitol is ground in the presence of 5 to 10 times its volume of buffer, the crude extracts would contain 70–140 mM concentrations of these sugars. Such concentrations are very inhibitory, as shown in Table 1 (see also Fig. 2a).

Influence of substrate concentration on inhibition by D*-mannitol*

Mannitol inhibited PAL activity less at higher substrate (L-phenylalanine) concentrations (Fig. 1). For instance, in the presence of 100 mM mannitol, the degrees of inhibition with 10 μM, 1 mM and 20 mM phenylalanine were 90%, 70% and 30% respectively. Mannitol apparently competes with phenylalanine for the free enzyme. However, Lineweaver–Burk plots for various concentrations of the inhibitor give straight lines that all intersect at a point to the left of the origin and above the 1/S axis (data not shown). Thus inhibition of PAL activity by mannitol is neither competitive nor uncompetitive, but is of the non-competitive type [9–11]: mannitol probably combines with the enzyme at a second site, wholly or partly different from the substrate-binding site; the presence of the inhibitor on the enzyme reduces its affinity for phenylalanine, but also the mannitol–PAL–phenylalanine complex breaks down more slowly than the PAL–phenylalanine complex does.

Nevertheless, the results illustrated in Fig. 1 show that when PAL has to be assayed in the presence of residual mannitol, rather high concentrations of phenylalanine should be used in order to reduce the degrees of inhibition. This is also illustrated by the shifts of the curves in Fig. 2a and b.

Reversibility of in vitro *inhibition of PAL activity by mannitol*

To find out if the inhibition is reversible, we ultrafiltered or diluted crude extracts containing inhibitory concentrations of mannitol (see Experimental). Relative to the crude extract free of mannitol (activity = 100%), activity of ultrafiltered crude extract free of mannitol was 70%. With 0.7 M mannitol added activities were respectively 0% and 70% for unfiltered and ultrafiltered crude extract. Hence, after ultrafiltration of mannitol-contami-

nated and mannitol-free crude extracts, PAL activity was about the same in both. This finding suggested that the *in vitro* inhibition of PAL was completely reversible and that a 100% recovery of PAL activity could be expected if a crude extract containing inhibitory concentrations of mannitol were diluted enough. Results of dilution experiments are shown in Fig. 2b. Low concentrations of mannitol (0–40 mM) seemed to slightly stimulate PAL activity (Fig. 2a and b), but this result was not found when we assayed purified PAL instead of a crude extract. Mannitol in the crude extract apparently also inhibits other enzymatic reactions, such as oxidation of phenols, which interfere with the conversion of phenylalanine into cinnamic acid. Such interfering enzymatic reactions were detected when we measured shifts in A. in the presence of 0.5 mM or 20 mM phenylalanine, using Zucker's spectrophotometric method [12]. A rather large increase in A at 290 nm for the blanks (phenylalanine free crude extracts) without mannitol, contrasted with almost no shift in A at 290 nm for similar blanks containing mannitol.

The relative PAL activity curves obtained after dilutions of the crude extract containing 200 mM mannitol show that PAL activity increases with the dilution factor (Fig. 2b). The curves of Fig. 2b and 2a are superimposable, and this demonstrates that *in vitro* inhibition of PAL by mannitol is completely reversible. As will be discussed later, the complete recovery of enzyme activity by diluting the crude extracts can be used to assay protoplasts and mesophyll cells suspended in 0.6–0.8 M mannitol.

DISCUSSION

As far as we know, the inhibition of PAL activity *in vitro* by mannitol or sorbitol has not been previously reported. Our data clearly show that measurements of PAL activity in crude extracts contaminated by hypertonic media may lead to ambiguous results. Concentrations of mannitol or sorbitol ranging from 70 to 150 mM are very likely to be present in such crude extracts. Even in the presence of 20 mM phenylalanine, such concentrations of sugars will reduce PAL activity by 10 to 99%.

Here we describe two methods for measuring PAL activity in protoplasts or isolated mesophyll cells as accurately as in leaf tissue. These methods are based upon the two following principles: 1. Mannitol or sorbitol can be used during isolation of the plant material but should be removed before it is ground; this can be achieved by substituting sucrose or salts for mannitol or sorbitol during the last washings. 2. If sucrose or salts cannot be used, PAL activity can still be carried out by a differential dilution procedure that will be described.

Procedure eliminating mannitol and sorbitol

One possibility is to use from the beginning hypertonic medium containing neither mannitol nor sorbitol. The osmotic pressure can be provided by sucrose or by salts that do not inhibit PAL *in vitro*. However, the classical procedures that give the best yield of viable protoplasts [8] or of protoplasts suitable for infection by viruses [13] involve a non-ionic osmoticum consisting of 0.6–0.8 M mannitol or sorbitol. Therefore we used these sugars during isolation of the mesophyll cells and protoplasts. For mesophyll cells, we followed the current version of the procedure of Takebe *et al.* [3]. The inhibitory sugars were removed by filtering the final suspension on nylon micromesh. The cells were then rinsed with 0.6 M sucrose, collected and assayed for PAL activity.

Because protoplasts are so small and fragile, they cannot be filtered on nylon micromesh. Final centrifugation of protoplasts is also difficult because in this dense medium the protoplasts do not pellet. Therefore, mannitol was eliminated by using one of the two procedures, derived from the methods of Meyer [14] and of Shepard and Totten [15]. In the first[14], salts are used as the plasmolyticum instead of the traditionally used sugars. However, the salt medium of Meyer is buffered and acidic, and PAL activity then has to be assayed at a pH lower than the optimum 8.8–9. The second method [15] uses low external osmotic conditions. The last washings of the protoplasts are carried out in 'Babcock milk-test like' bottles containing 0.45 M sucrose. Intact protoplasts float to the top of the solution during centrifugation and are easily withdrawn with a Pasteur pipette.

Differential dilution

A differential dilution procedure can be used to measure PAL activity of single cells or protoplasts in the presence of residual amounts of mannitol, sorbitol or other inhibitory plasmolytica. It is based on the complete reversibility of the *in vitro* inhibition, as illustrated in Fig. 2. In the dilution experiments graphed in Fig. 2b, 200 mM mannitol was present in the crude extract for one hr at 4° before the various dilutions were performed. After 4-fold dilution, the residual (50 mM) mannitol was no longer inhibitory for PAL incubated with 0.5–20 mM phenylalanine. Let us take the example of a 5 ml pellet of protoplasts, containing residual medium, that is to be assayed. If the osmoticum consisted of 0.7 M mannitol or sorbitol and if the pellet was ground in 20 ml of buffer, the crude extract would contain residual sugars at a concentration less than $0.7\ M \times 5/(20 + 5) = 140\ mM$. According to the curves of Fig. 2b, a solution obtained by two fold dilution of this crude extract and incubated with 20 mM phenylalanine should allow accurate determination of PAL activity. To confirm the result, another dilution, for instance 3-fold, should be performed and the resulting solution incubated in the same conditions. The observation that the measured enzymatic activities are in the same ratio as the dilutions (in the present case 2/3) is reliable evidence that the residual amounts of sugars in even the less diluted solution are not inhibitory.

Elimination of mannitol or differential dilution enabled us to measure PAL activities of protoplasts or isolated mesophyll cells easily. In order to compare these samples with the starting leaf tissue, we express enzyme activities relative to the same amount of soluble proteins present in the crude extracts. We found PAL activity of the same order of magnitude in mesophyll cells, protoplasts, tobacco leaves, provided the external osmotic pressure was applied for no more than 12 hr (M. Kopp *et al.*, unpublished).

EXPERIMENTAL

Conditions of plant culture. We used the two first fully expanded leaves from the top of *Nicotiana tabacum* plants, var. Samsun NN, grown in an air-conditioned greenhouse at 22° ± 2°. Supplementary lighting was supplied by Na lamps 16 hr per day at a bench-level intensity of $1–3 \times 10^4$ lx.

Isolation of mesophyll cells. Mesophyll cells were isolated using procedure of ref. [3]. To remove mannitol, the cell suspension was filtered on nylon micromesh (50 μm) under a slight vacuum and then rinsed with 0.6 M sucrose before being collected.

Isolation of protoplasts. These were isolated using either the two-step procedure of ref. [3] or the one-step method of ref. [7]. To remove mannitol, the final centrifugation of the protoplasts was performed in a medium in which the non-ionic osmoticum was replaced by a salt medium [14]. In some cases, the one-step method [7] was used under low external osmotic (0.45 M mannitol) conditions, and mannitol was removed by a final centrifugation on 0.45 M saccharose in 'Babcock milk-test like' bottles. These were made by fusing a 5 cm piece of a graduated 10 ml pipette to the top of a 30 ml bottle-shaped centrifuge tube. Protoplasts floated to the top of the soln during the 3 min centrifugation at 650 rpm and were then concentrated to a vol. of 1–3 ml.

Extraction of PAL and measurement of its activity. The extraction of PAL from tobacco leaves has already been described [1]. Isolated mesophyll cells or protoplasts were ground in a mortar in the presence of quartz sand and 0.1 M ice-cooled borate buffer, pH 8.8, containing 15 mM mercaptoethanol. The mixture was filtered through a double layer of cheesecloth and the filtrate was centrifuged at 20000 *g* for 30 min. For crude extracts incubated with 0.1 mM phenylalanine, enzymatic activity was measured radiochemically [1]. For those incubated in the presence of 0.5 mM or 20 mM phenylalanine, PAL activity was determined according to Zucker's spectrophotometric assay [12]. Proteins were measured as in ref. [16].

Reversibility of the mannitol inhibition of PAL. The crude extract was prepared from tobacco leaves with 0.1 M borate buffer, pH 8.8. Ultrafiltration was performed in an Amicon cell equipped with a PM 30 membrane. Buffer was added in several lots during ultrafiltration in order to completely eliminate mannitol. After ultrafiltration and before an aliquot was assayed for PAL activity, the concentrate was diluted with borate buffer and the vol. adjusted to the initial vol. of the crude extract. PAL activity of the various samples was measured as described in the legend of Table 1.

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