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MULTIPLICITY OF SPINACH ROOT PHENOLASE AND ITS MONOPHENOLASE ACTIVITY

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; phenolase; monophenolase; enzyme multiplicity; root.

Abstract—Soluble phenolase of spinach roots is present in multiple forms, none of which is electrophoretically identical with those detected in the chloroplasts. Three out of four forms can oxidize monophenols, in contrast with the leaf enzymes. The enzyme activity seems to be present largely in the bound form.

INTRODUCTION

Spinach phenolase has been known to exist in both aerial and terrestrial organs. The enzyme in leaves, predominantly located in the chloroplasts [1], is present in an inactive latent form [1–11] and lacks monophenolase (hydroxylating) activity, i.e. it exhibits only *o*-diphenolase (quinone-forming) functions [1, 4, 6]. In seedlings, the root phenolase is 500-fold higher than the total activity in green parts and is devoid of latency [12]. Its enzyme form and monophenolase activity have not, however, been examined, and further studies have now been carried out.

RESULTS AND DISCUSSION

Fig. 1 shows the polyacrylamide gel electrophoresis patterns after the gels were incubated with DOPA (for *o*-diphenolase) and tyrosine (for monophenolase) in the presence of a soluble fraction prepared from the roots of water-cultured seedlings and field-grown plants at various developmental stages. The pattern obtained with an extract from the chloroplasts [7–11] is also included. The results are summarized as follows.

(1) For *o*-diphenolase, roots possess four enzyme forms in all (named 1–4 in order of their electrophoretic mobility), none of which can be identified

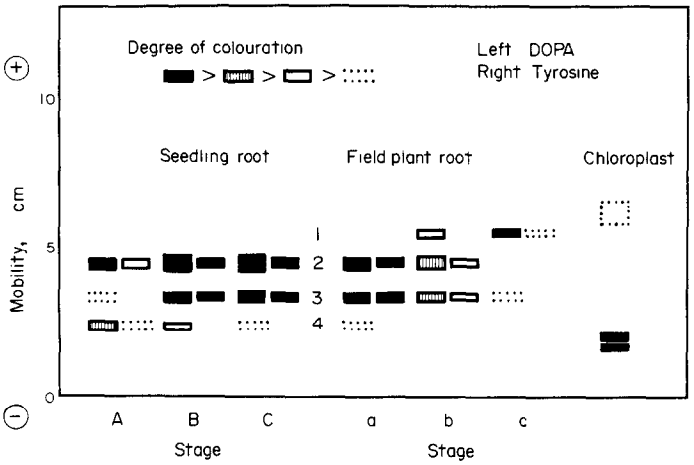

Fig. 1. Polyacrylamide gel electrophoresis patterns of phenolase (*o*-diphenolase and monophenolase) in spinach roots and chloroplasts. 50 μl of 30 000 *g* supernatant of root macerate (g/ml medium) and an extract from the chloroplasts were applied to the column, and electrophoresis was run for 30 min at 2 mA/tube. The activities of *o*-diphenolase and monophenolase were detected by incubating the gels for 20 min in L-DOPA and for 5 hr in L-tyrosine, both as saturated solutions in 0.2M Pi buffer, pH 6.8, respectively, at room temperature. Stages: A, seedlings with radicles of *ca* 1 cm in length; B, with young roots (4–5 cm) in which vascular system and many fine root hairs had clearly developed; and C, with elongated rigid main roots (10–12 cm) branching many lateral roots. From the field-grown plants, more or less rigid roots were collected 25, 35 and 60 days after sowing (*ca* 3, 5 and 10 cm in length, respectively), and they are designated as roots at stages a, b and c, respectively.

with the three enzyme forms in the chloroplasts. With other *o*-diphenols, such as catechol and caffeic acid, the same patterns were obtained. Unlike previous results showing a single enzyme form on elution from a Sephadex column and electrophoresis on a Cellogel plate [12], the multiplicity of the root phenolase was established.

(2) Prolonged incubation (5 hr or more) revealed that forms 2–4 could form a strong colour with the monophenol. Form 1 did not exhibit this activity. It was confirmed that the chloroplast phenolase is unable to oxidize monophenols [4]. *p*-Coumarate did not colour the gels even after overnight incubation.

(3) Enzyme forms with slower mobility detected at earlier stages (the pattern at stage a of the field plants seems to correspond to that at stages B and C of the seedlings) tend to be replaced by the forms with greater mobility. Similar change during development was reported in the multiple enzymes of the chloroplasts of this plant [4]. In the roots at stage C, form 1 became predominant, being deficient in monophenolase activity like the chloroplast enzymes.

Distribution of the enzyme activity in tissue macerate was subsequently examined (Table 1). Only *ca* 6% of the total activity was recovered in the soluble fraction (30 000 *g* supernatant), while most activity

Table 1. Distribution of phenolase activity in root tissue macerate

	Fraction No.	Activity (%)
1	30 000 *g* supernatant of 1st macerate*	3.7
2	30 000 *g* supernatant of 2nd macerate*	1.2
3	30 000 *g* supernatant 3rd macerate*	0.5
4	30 000 *g* supernatant 4th macerate†	0.3
		(5.7)
5	90 *g* supernatant of 1st washing	19.4
6	90 *g* supernatant of 2nd washing	4.4
7	1000 *g* supernatant of 3rd washing	1.3
8	3000 *g* supernatant of 4th washing (after 2 days standing)	0.1
		(25.2)
9	Final pellet	69.1

Activity was estimated colorimetrically with catechol as substrate.

*, † Macerations were carried out with a pestle and mortar, and in a mixer at top speed for 3 min, respectively.

Table 2. Oxygen uptake by several fractions with mono- and *o*-diphenols

Substrates	Oxygen uptake* by fractions† 1‡	5	9
Monophenols			
Methylphenol	1.26 (26)	3.37 (16)	19.1 (45)
L-Tyrosine	0.32 (7)	3.89 (18)	14.6 (34)
p-Coumarate	0 (0)	0 (0)	2.6 (6)
4-Hydroxybenzoate	0 (0)	0 (0)	0 (0)
o-Diphenols			
Catechol	1.25 (25)	10.9 (51)	31.4 (74)
L-DOPA	4.93 (100)	21.2 (100)	42.5 (100)
Caffeate	1.21 (25)	5.1 (24)	23.5 (55)
Protocatechuate	0 (0)	0 (0)	0 (0)

*μl O_2/10 min/g tissue.

†See Table 1.

‡35–55% ammonium sulphate precipitate, dialysed against 10 mM Pi buffer, pH 6.8.

The values in parentheses represent the relative oxidation rate, when DOPA was taken as 100.

(*ca* 70%) was detected in precipitates obtained on low speed centrifugation (fraction 9), being devoid of smaller particles by repeated washings.

All the fractions tested (fractions 1, 5 and 9 in Table 1) could oxidize certain monophenols besides *o*-diphenols (Table 2). Fraction 9 showed, however, higher affinity for certain phenolic substances and could convert *p*-coumarate to caffeate (the product was identified using a system with ascorbate [13]).

Incubation of this fraction with 2% Triton X-100 (pH 6.8) or with a mixture of hydrolytic enzymes (2% each of cellulase, hemicellulase and pectinase, pH 5.5), at 30° up to 6 hr did not release the enzyme.

EXPERIMENTAL

Materials. Spinacia oleracea var. Minsterland was used. Seeds were soaked for 2 days at 10–17° to remove germination inhibitors (e.g. oxalates [14]). They were sown on a layer of moist gauze, which was laid on a dish filled with H_2O and seedlings grown for 10–12 days. Field plants were cultivated in our campus from mid-September until late December.

Electrophoresis. Fresh roots at various developmental stages were thoroughly crushed with a pestle and mortar using a sufficient amount of quartz sand in 50 mM Pi buffer pH 6.8 (1 g in 1 ml) and the brei squeezed through silk. The filtrate was centrifuged at 30 000 *g* for 20 min, the supernatant dialysed against the electrophoresis buffer (1M Tris–glycine buffer, pH 8.3), and the dialysate, after mixing with an equal vol. of 1M sucrose, was used for electrophoresis on polyacrylamide gel (7.6%).

The soln of the chloroplast phenolase was prepared from the Me_2CO powder of the chloroplasts which had been pre-treated with Triton X-100 to activate the latent enzyme and render it soluble, as previously described [7–11].

Fractionation. The fractions in Table 1 were prepared as follows. 16 g seedling roots at stage B (see text) was macerated in 70 ml 50 mM Na-Pi buffer at pH 6.8 with a pestle and mortar and the brei centrifuged at 30 000 *g* for 20 min. The pellet was taken up in 50 ml of the same buffer, re-macerated and centrifuged. These procedures were repeated. The ppt., suspended in 100 ml of the buffer, was subjected to further homogenization in a mixer at top speed for 3 min and centrifuged as above. Four supernatant fractions (fractions 1–4) thus obtained were used for analysing soluble enzyme. The pellet was thereafter washed three times by thorough suspension in 100 ml of the buffer and by keeping smaller particles in supernatants (fractions 5–7) of low speed centrifugation at successive 90, 90 and 1000 *g* for 5 min. After standing the suspension of the last ppt. taken up in buffer for 2 days, centrifugation at 3000 *g* for 10 min gave fraction 8 as the supernatant. The final pellet was suspended in 50 ml of the buffer, and the suspension represented as fraction 9.

Assay of enzyme activity. Enzyme activity was assayed either by the colorimetric method for *o*-diphenolase [7–11], or by manometry for monophenolase, where 1 ml of enzyme preparation (soln or suspension) in 10 mM Pi buffer, pH 6.8, was placed in the flask and 0.5 ml of 1.25×10^{-2} M substrate in 50 mM of the same buffer in the side arm and the initial velocity determined within 4 min.

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