Phytochemistry, 1976, Vol. 15, pp. 1845–1847 Pergamon Press. Printed in England.

PHENOLASE OF SPINACH ROOTS

MITSUHIKO SATÔ
Department of Biology, Faculty of Science, Tokyo Metropolitan University, Setagaya-ku, Tokyo, 158 Japan

(*Revised received* 20 *May* 1976)

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

Abstract—Phenolase activity is not found in germinating spinach embryos, but it appears in the radicles when the vascular tissues have developed, and then increases progressively. Unlike the two phenolases detected earlier in the chloroplasts, the root enzyme is a single protein with higher MW occurring both in 3000 *g* precipitate and 28000 *g* supernatant fractions. The phenolase in 3000 *g* fraction is not activated by treatment with detergents and trypsin. The enzyme is contained mainly in xylem parenchymatous cells adjacent to primary vessels. It also occurs to a lesser degree in the dermal parts, including epidermis and cortex. Similar tissue-level distribution patterns of this enzyme are also observed in the roots of other angiosperms, especially in Compositae.

INTRODUCTION

The wide distribution in the plant kingdom of a copper-containing enzyme, phenolase (*o*-diphenol:O_2 oxidoreductase), is now well established [1,2] and some of its roles in plants have recently been hypothesized [3]. The information on this enzyme in higher plants came largely from analyses of aerial organs, leaves and fruits, whilst only a restricted knowledge is available for the enzyme in roots [4].

Spinach chloroplasts contain two enzymes with catecholase activity [5] which may be interconverted [5,6]. The main purposes of the present study are to characterize the properties of root phenolase of spinach with special reference to its comparison with the enzymes present in the chloroplasts.

RESULTS

Comparison of phenolase activity in various parts of spinach seedlings

The catecholase activity in several parts of 20-day-old seedlings of spinach is shown in Table 1. As compared with upper green part of cotyledon, a high activity was detected in roots, based upon both one plant and fr. wt (*ca* 500- and 6000-fold, respectively).

Appearance and increase of enzyme activity during root development

The enzyme activity of the root was examined at several developmental stages (Table 2). No activity could be observed in the imbibing embryo (stage I) and in young seedlings with radicle of *ca* 5 mm in length (stage II). The possibility that the enzyme exists in a latent form, as is the case for leaf chloroplast phenolase [5], was tentatively ruled out, since activity did not increase when the tissue brei from these stages was treated for 5–6 hr with 1% Triton X-100, 0.1% and 1% Na-deoxycholate or trypsin (0.8 mg in 1 ml), all of which are known as reagents for breaking the latency of this enzyme [5,7]. A distinct activity appeared in radicles of the next stage (stage III), by which time vascular tissues and many fine root hairs clearly developed. Subsequently, the cotyledons emerged from the seed coat and the main root continued to elongate (stage IV), and finally rigid lateral roots developed (stage V). The enzyme activity increased successively up to 13.5-fold from stage III to V.

Table 1. Comparison of phenolase activity in several parts of 20-day-old spinach seedlings

Part		Relative activity Per plant	Per fr. wt
Cotyledon	upper green part	1.0	1.0
	basal part	5.1	10.1
Hypocotyl		18.4	41.1
Root		543.0	5970.0

Intracellular localization of the enzyme

Intracellular localization of the enzyme in root from stage III to V is shown in Table 3. It seems that some fluctuations occur in the course of the root development. The activity detected in the 3000–11000 *g* fraction from stage III decreased at the following stage and that in the 3000 *g* fraction (plastid fraction) correspondingly increased. This may be explained by supposing that the former fraction contains proplastids which could not be sedimented by 3000 *g* centrifugation. From stage IV to V a decrease of phenolase activity in the 3000 *g* fraction

Table 2. Appearance and increase of phenolase activity during root development

Stage	Organ	Days	Relative activity Per plant	Per fr. wt
I	Imbibing embryo	0	—	—
II	Radicle (5 mm)	3	—	—
III	Radicle (1 cm)	5	1.0	1.0
IV	Root (5 cm)	10	2.6	1.7
V	Root (10 cm)	20	13.5	7.0

Table 3. Intracellular distribution of phenolase activity in spinach root

Fraction	Activity* at stage III	IV	V
3000 *g* ppt.	35	51	38
11000 *g* ppt.	42	21	15
28000 *g* ppt.	9	11	6
28000 *g* supernatant	14	17	42

* Expressed as % of the total.

seem to be accompanied by an increase in the 28000 *g* supernatant fraction. Generally speaking, however, the root plastids occupy 30–50% of the phenolase activity in the cells.

Absence of activation by detergents or trypsin of plastid phenolase in 3000 g *fraction*

The 3000 *g* fraction was subjected to treatment with several detergents and trypsin (Table 4). A slight increase of the total activity in the digest was obtained with Na-deoxycholate, but the other reagents were rather inhibitory. Liberation of the enzyme was much smaller than in the case of the chloroplasts where 100% of activity could be recovered in the 25000 *g* supernatant after treatment with Na-deoxycholate for 1 hr [5]. It has been reported that a proteolytic enzyme, trypsin, could activate the latent phenolase of spinach intact and broken chloroplasts by *ca* 10- and 40-fold, respectively [7]. No enhancement of activity by trypsin, however, could be observed toward phenolase in 3000 *g* fraction of the roots.

Single enzyme form

The form of the enzyme in the 28000 *g* supernatant was examined by TLG filtration on a Sephadex G-200 plate and cellogel electrophoresis, and it was confirmed that the enzyme is present in a single form, irrespective of the developmental stages. The enzyme appeared at the void volume from the Sephadex G-200 column so that it seems to be a higher MW protein than those found in the chloroplasts. The phenolase obtained in the 28000 *g* fraction by treating the 3000 *g* fraction with 0.1% Na-deoxycholate for 2 hr was a single protein with the same mobility as above.

Distribution of the enzyme in the root tissues

The activity of the enzyme per unit length of the root axis of mature spinach is constant and independent of the part, despite the fact that the fr. wt decreases continuously from base to tip and hence the activity per *g* fr. wt increases (Table 5). The activity in part 3 from which many lateral roots branch endogenously is slightly higher than in the other parts, and the total activity occupied by these lateral organs is greater than that in main root, although the fr. wt of the former is only one-third of the latter. These facts might be best accounted for by assuming that the enzyme is concentrated in a certain tissue(s) running longitudinally through the root.

On examining histochemically the transverse and longitudinal sections of various parts, it was found that the red coloration due to the reaction of the enzyme with substrate (catechol–sulphanilic acid solution) appeared strongly at first in cells adjacent to primary vessels, i.e. in xylem parenchymatous cells. The coloration developed, though to a lesser degree, also in dermal parts including epidermis and outer layers of cortex.

Table 4. Effect of detergents and trypsin on phenolase activity in root plastids

Reagents	Relative activity of Digest* (a)	Supernatant† (b)	Dissociation rate (b)/(a) × 100
1% Na-dodecylsulphate	51	28	(55)
1% Na-deoxycholate	139	75	54
1% Triton X-100	57	34	(60)
1% Tween 80	66	36	(55)
Trypsin (0.8 mg/ml)	102	33	32
Control‡	100	6	6

* Plastid suspension after standing for 2 hr at 15° with reagent dissolved in 10 mM Pi buffer pH 6.8. †Supernatant of the digest at 28000 *g* for 30 min. ‡The plastid suspension stood for 2 hr in the same buffer. The values in parentheses show no accurate dissociation rate, because the enzyme was partly inhibited.

Table 5. Distribution of phenolase activity along root axis of matured spinach

Organ	Part	Distance from tip (cm)	Number of lateral root	Fr. wt (mg)	Relative activity Per part	Per *g* fr. wt
Stem		13–12		218	1.29	2.56
Main root	(1)	12–10	3	434	1.00	1.00
	(2)	10–8	5	278	1.10	1.71
	(3)	8–6	18	166	1.27	3.32
	(4)	6–4	5	87	1.12	5.58
	(5)	4–2	6	49	1.17	10.3
	(6)	2–0	0	23	1.05	19.8
				1040	6.74	
Lateral roots				306	8.75	19.2

No activity could be detected in cambium, phloem and secondary tissues. Since the epidermis of matured roots is often missing, the distribution pattern of the enzyme was further examined in young seedlings from stage III to V. The clear coloration of the xylem cells around vessels was again confirmed, and the dermal part, especially epidermis with root hairs, strongly coloured after longer standing.

In order to examine the tissue-level distribution of angiosperm root phenolase, some exploratory observations were run using several plants including those collected in early March. The plants may be divided into three groups. The first group is completely lacking in the activity, the second possesses it only in the dermal part, and the last, as is the case for spinach, both in the dermal part and xylem parenchymatous cells around the primary vessels. It is of interest that all the members of the Compositae so far tested, belong to the last group.

EXPERIMENTAL

Materials. Seeds of spinach (*Spinacia oleracea* var. World) were soaked for 2–3 days in H_2O at 5–10° to remove germination inhibitors [8]. The seedlings with radicle of *ca* 1–2 mm in length were placed on several sheets of moistened paper laid on Petri dishes and grown at 15° (optimum) in a growth cabinet (12-hr day, 3000 lx). The roots of matured spinach were supplied from a farm in Hayano, of the Kanagawa prefecture. TLG on Sephadex [5], cellogel electrophoresis [6] and Sephadex column chromatography [5,6] have already been described.

Estimation of enzyme activity. A colorimetric method [5] was employed. A mixture of 0.5 ml of enzyme sample, 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° and the red colour was estimated at 500 nm.

Cellular fractionation. Roots were macerated with pestle and mortar in 5 vol. of a medium consisting of 0.4 M sucrose, 0.05 M Tris-HCl buffer pH 7.8 and 0.01 M NaCl. The macerate was filtered through silk and the filtrate was subjected to successive centrifugation. (Since it has been pointed out that during tissue maceration the endogenous phenolic compounds cause the adsorption of soluble phenolase to particle fractions to bring about artefacts [9,10], an EtOH extract from the roots at stages III and V was tentatively examined with $FeCl_3$, phosphomolybdic acid and diazotized benzidine [11], and it was found that no significant amount of phenolic substances is contained in roots of spinach seedlings, although a slight accumulation was observed in the roots of later stage.) The fractions used in Tables 1, 2 and 5 for estimating the enzyme activity were obtained as follows. The tissues were thoroughly crushed in the above medium with quartz sand and the macerate was transferred to a test tube followed by standing for several min to sediment the sand and larger cell debris. The upper slightly turbid suspension thus obtained was directly used as enzyme sample, with a reference which contained the same vol. of suspension, and water instead of catechol.

Histochemical observation. Surfaces of root sections, *ca* 0.5 mm in thickness, was softly contacted with a filter paper which had been immersed in a bufferized catechol-sulphanilic acid solution, and the red colour developing within 1–3 min was observed under a light microscope.

Acknowledgements—The author would like to thank Prof. M. Hasegawa for his useful criticism throughout this study. Thanks are also due to Drs. M. Ono and K. Ohnishi of our faculty for their taxonomic guidance.

REFERENCES

1. Scandalios, J. G. (1974) *Ann. Rev. Plant Physiol.* **25**, 225.
2. Stafford, H. A. (1974) *Ann. Rev. Plant Physiol.* **25**, 459.
3. Marbach, I. and Mayer, A. M. (1974) *Plant Physiol.* **54**, 817.
4. Taneja, S. R. and Sachar, R. C. (1974) *Phytochemistry* **13**, 2695.
5. Satô, M. and Hasegawa, M. (1976) *Phytochemistry* **15**, 61.
6. Satô, M. (1976) *Phytochemistry* **15**, 1665.
7. Tolbert, N. E. (1973) *Plant Physiol.* **51**, 234.
8. Hasegawa, M., Iwakura, Y. and Nabetani, A. (1956) *J. Chem. Soc. Japan* **77**, 1320.
9. Sanderson, G. W. (1964) *Biochim. Biophys. Acta* **92**, 622.
10. Takeo, T. (1965) *Agr. Biol. Chem.* **29**, 558.
11. Satô, M. (1966) *Phytochemistry* **5**, 385.