Phytochemistry, 1976, Vol. 15, pp. 707–710. Pergamon Press. Printed in England.

ACTIVITY OF ENZYMES INVOLVED IN LIGNIN BIOSYNTHESIS IN SWEDE ROOT DISKS

MICHAEL J. C. RHODES, A. CHARLES R. HILL and
LEONARD S. C. WOOLTORTON
A.R.C. Food Research Institute, Colney Lane, Norwich, NR4 7UA

(*Revised received* 15 *December* 1975)

Key Word Index—*Brassica napo-brassica*; Cruciferae; swede root; ageing; enzyme changes; *O*-methyl transferase properties; effects of ethylene.

Abstract—The activity of nine enzymes involved in the biosynthesis of lignin precursors has been studied during the ageing of swede root disks in the presence and absence of ethylene. Peroxidase, aromatic alcohol dehydrogenase and phenylalanine transaminase show very little change in activity during ageing under either ageing condition. *O*-methyl transferase, shikimate dehydrogenase and ferulyl CoA reductase show only a 2–3 fold increase on ageing and are relatively insensitive to ethylene treatment. A third group (comprising phenylalanine ammonia lyase, cinnamic acid-4-hydroxylase and hydroxycinnamate CoA ligase) show 20–30 fold increase on ageing and are most sensitive to ethylene treatment. Phenylalanine ammonia lyase and cinnamic acid-4-hydroxylase behave very similarly in respect of their time course of ageing and in their responses to metabolic inhibitors such as cycloheximide, puromycin and actinomycin D. In addition the properties of the *O*-methyl transferase of swede root tissue are described.

INTRODUCTION

It has been shown that disks of swede roots aged for 24 hr develop a layer of a lignin-like substance [1] which is restricted to the walls of the cells on the cut surface and the adjacent layer. It has been shown that treatment of the disks during ageing with ethylene stimulates the formation of this lignin material [2] and enhances the increase in activity of phenylalanine ammonia lyase [3]. The properties of other enzymes involved in phenylpropanoid biosynthesis in aged swede disks have been described in previous publications [3–7], and the present paper includes some properties of the *O*-methyl transferase of swede disks. The changes in the activity of nine enzymes involved in the pathway of phenolic biosynthesis from simple sugar precursors during the ageing process and the effects of inhibitors of RNA and protein synthesis on a few important members of this group of enzymes will be described.

RESULTS

Properties of the O-methyl transferase

The *O*-methyl transferase activity of swede root tissue has a pH optimum at 7 (pHs for half maximal activity at 5.4 and 8.1). The enzyme shows high affinity for its substrates, caffeic acid and *S*-adenosylmethionine (apparent K_m values of 9.2×10^{-5} M and 1.5×10^{-5} M respectively). The enzyme is specific for methylation in the *meta* position of caffeic acid. 5-hydroxyferulic acid can act as the acceptor but the rate of methylation is lower than with caffeic acid. *p*-Coumaric, ferulic and sinapic acids and coniferyl alcohol all cause some inhibition of the enzyme. Double reciprocal plots of the rate of enzyme reaction against substrate concentration at different inhibitor concentrations showed that the inhibition by the phenolic compounds was non-competitive and K_i values of 3.5, 4.0, 2.5 and 2.0 mM were obtained respectively for *p*-coumaric acid, ferulic acid, sinapic acid and coniferyl alcohol.

Changes in enzyme activity during ageing

Figure 1 shows the time course of changes in the activity of four enzymes during the ageing of swede root disks in the presence of ethylene. Two of the enzymes, phenylalanine ammonia lyase (PAL) and cinnamic acid-4-hydroxylase (CAH) show very similar patterns of increase during ageing from very low initial values. The other two enzymes, shikimate dehydrogenase (SDH) and *O*-methyl transferase (OMT) show a different pattern. There is a steady rise in OMT after a lag of 4 hr while in the case of SDH there is an initial fall in activity followed by a rise of *ca* 2-fold. The overall increase in activity is very much smaller in the case of SDH and OMT compared with PAL and CAH.

Table 1 shows the relative levels of activity of nine enzymes involved in phenylpropanoid biosynthesis in initial disks, in disks aged for 24 hr in air and in disks aged for 24 hr in air containing 8 ppm ethylene. The enzymes show considerable differences in their pattern of changes during ageing. Some enzymes, transaminase, aromatic alcohol dehydrogenase and peroxidase show no significant increase in total activity and no response to ethylene treatment. Another group, including SDH, OMT and ferulyl CoA reductase increase only 2–3 fold during the 24 hr period and their increases are only slightly stimulated by ethylene treatment. The third group, including PAL, CAH and hydroxycinnamate CoA ligase, are initially present only at very low levels, in-

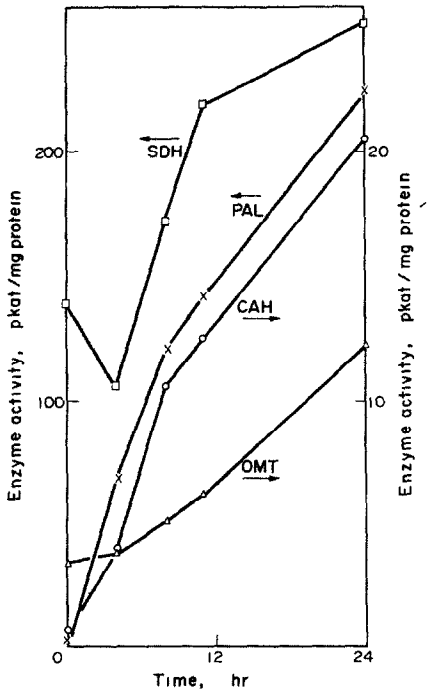

Fig. 1. Time course of changes in enzyme activity during the ageing of swede root disks in the presence of 8 ppm ethylene. □ Shikimate dehydrogenase (SDH); △ *O*-Methyltransferase (OMT); ○ Cinnamic acid-4-hydroxylase (CAH); × Phenylalanine ammonialyase (PAL). Left hand scale refers to SDH and PAL and right hand scale to CAH and OMT.

crease 20–30 fold during ageing, and the increase is stimulated 2–5 fold by ethylene treatment. The stimulation by ethylene is greatest in the case of PAL.

Effect of inhibitors on changes in enzyme activity on ageing

Table 2 shows the effect of inhibitors of RNA and protein synthesis on the induction of three enzymes, PAL, CAH and OMT during ageing. Separate experiments showed that cycloheximide will inhibit the incorporation of leucine-^{14}C into a TCA insoluble fraction at concentrations above 1 μg/ml (3.6×10^{-6} M) and that while it will inhibit the induction of the three enzymes at concentrations above 2 μg/ml (7.2×10^{-6} M) it has no effect on the assay of these enzymes even when added to assay mixtures at concentrations up to 100 μg/ml (3.6×10^{-4} M). Table 2 shows that in this particular experiment PAL increased 23 fold and CAH increased 22 fold, while OMT increased only 3.2 fold during the 24 hr ageing period in the presence of ethylene. Cycloheximide at 2 μg/ml almost completely prevented increase in activity of the three enzymes. If the inhibitor was removed from the ageing disks after 11 hr of treatment and the disks returned to inhibitor-free medium for a further 13 hr then the inhibition of the increases in enzyme activity was reversed. The degree of reversal was almost the same for PAL and CAH at 48 and 56% respectively, while that for OMT was somewhat lower at 30%. Puromycin, at 50 μg/ml is a much less effective inhibitor than cycloheximide and caused only a 48% inhibition of the increases in PAL and CAH activity and only a 14% inhibition of the increase in OMT. The inhibitor of RNA synthesis, actinomycin D (5 μg/ml), caused an 80% inhibition of the increase in PAL and CAH activity but only a 38% inhibition of that in OMT. Attempts to remove actinomycin D after 11 hr by returning disks to inhibitor free medium for a further 13 hr did not result in a reversal of this inhibition.

DISCUSSION

Of the nine enzymes of phenylpropanoid biosynthesis studied, only three (PAL, CAH and *p*-coumarate CoA ligase) showed a large increase (20–25 fold) in activity on ageing of swede root disks. The other enzymes studied showed much smaller increases in activity. Swede tissue has a very high activity of peroxidase, an enzyme known to show a high degree of heterogeneity [8] and it may be that although there are no major changes in total peroxidase activity in any of the treatments there may be underlying changes in isoenzymic forms possibly specifically involved in phenylpropanoid biosynthesis. Evidence for changes in the isoenzymic compositions of aromatic ADH's during ageing has been previously published [9].

The role of ethylene in the changes of enzyme activity during ageing is unclear since disks produce significant amounts of the lignin-like product and show major changes in the activity of several enzymes even in the absence of exogenous ethylene. Exogenous ethylene amplifies these changes in enzyme activity and particularly affects PAL and to a lesser extent CAH and *p*-coumarate CoA ligase. It has previously been shown that cutting and ageing swede root disks leads to an increased

Table 1. The relative enzyme activities of swede root extracts prepared from initial disks, disks aged in air and in air containing 8 ppm C_2H_4

	Relative activity (initial disks = 1)	
	Disks aged in air + 8 ppm C_2H_4	Disks aged in air
1. Shikimate dehydrogenase	1.8	1.8
2. Phenylalanine transaminase	1.0	1.2
3. Phenylalanine ammonia lyase	22.5	4.4
4. Cinnamic acid-4-hydroxylase	22.8	12.7
5. *O*-Methyl transferase	3.7	2.3
6. *p*-coumarate CoA ligase	22.4	9.5
7. Ferulyl CoA reductase	2.8	—
8. Coniferyl alcohol NADP oxidoreductase	1.2	—
9. Peroxidase	1.4	1.1

Table 2. The effect of inhibitors on changes in enzyme activity during ageing of swede root disks

	(pkat/mg protein) Values in brackets are the activities relative to the activity in initial disks which is taken as 100		
	PAL	CAH	OMT
Experiment A			
Initial disks	37 (100)	12 (100)	30 (100)
Disks aged for 24 hr	870 (2350)	260 (2170)	96 (320)
Disks aged for 24 hr in presence of 1 μg/ml cycloheximide	58 (157)	19 (158)	20 (67)
Disks aged for 11 hr with 1 μg/ml cycloheximide and transferred to a medium free of cycloheximide for a further 13 hr	440 (1190)	113 (942)	50 (161)
Experiment B			
Initial disks	30 (100)	9 (100)	20 (100)
Disks aged for 24 hr	780 (2600)	235 (2610)	102 (510)
Disks aged for 24 hr, in presence of 50 μg/ml puromycin	420 (1400)	130 (1440)	90 (450)
Disks aged for 24 hr in presence of 5 μg/ml actinomycin D	190 (633)	50 (556)	71 (355)

PAL = phenylalanine ammonia lyase; CAH = cinnamic acid-4-hydroxylase; OMT = *O*-methyltransferase

rate of ethylene production and that treatment of undamaged swede roots with ethylene leads to a large increase in PAL activity [3]. These results suggest that the response in the absence of added ethylene could be due to endogenous ethylene produced in response to wounding but this is as yet unproven.

Cycloheximide [9] inhibits amino acid incorporation and prevents the increases in PAL, CAH and OMT activity during ageing. Both puromycin, an inhibitor of ribosomal protein synthesis [10] and actinomycin D, an inhibitor of DNA directed RNA polymerase [11] inhibit the increase in activity of the three enzymes but are less effective than cycloheximide. No evidence was found for the presence of proteinous inhibitors and inactive forms of the enzymes in extracts of initial disks and the simplest hypothesis is that RNA and protein synthesis are involved in the observed increases in enzyme activity. It is interesting that two enzymes (PAL and CAH), which are known to be located in different cell organelles, show marked similarities in both the degree and time courses of changes in ageing and in their responses to the addition and removal of inhibitors from the medium in which the disks were aged. In swede roots, the bulk of the PAL is soluble while nearly all the CAH is located in microsomal particles [12]. However, some PAL is bound to the same particles as CAH [13] and cannot be removed simply by washing [12]. PAL and other enzymes of the phenylpropanoid biosynthetic pathway *in vivo* may be loosely associated with a lipo-protein membrane system of which CAH forms an integral part.

There have been a number of recent studies of the changes in enzyme activity in plant tissues under conditions in which the biosynthesis of phenolic compounds has been stimulated. During the growth of cell suspension cultures of *Glycine max* in the light, [14] there were large and concomitant increases in the activity of PAL, CAH and *p*-coumarate CoA ligase associated with enhanced flavonoid biosynthesis. In the same period, the activity of the other enzymes involved in the pathway of flavonoid biosynthesis remained unchanged. Similarly, a large increase in PAL and CAH occurs during the ageing of potato tuber disks in the light [15] whereas aspartate-phenylpyruvate transaminase, peroxidase and SDH increased only to a small extent. A large increase in *p*-coumarate CoA ligase is also found in potato tuber disks aged in the light [17]. These results taken with those presented here for swede root disks indicate that in a number of plant tissues PAL, CAH and the *p*-coumarate CoA ligase play an important role in the overall regulation of the pathway of biosynthesis of phenylpropanoid compounds.

EXPERIMENTAL

Preparation of disks of swede roots and conditions of ageing of the disks are as described in ref. [3]. Various inhibitors were included in the incubation solns at the concentrations stated in the text. Methods of disk extraction and the assay of PAL [3], CAH [6], hydroxycinnamate CoA ligase [4], hydroxycinnamyl CoA reductase [5] and the aromatic alcohol dehydrogenase [7] are as described elsewhere. Peroxidase [18], shikimate dehydrogenase [19] and phenylalanine transaminase [20] were assayed by standard methods. *O*-methyl transferase was assayed on extracts prepared in the standard manner in an assay based on that of ref. [21]. The enzyme was incubated in a mixture containing 0.1 M HEPES pH 7, 1 mM caffeic acid, 0.2 mM *S*-adenosyl methionine-1-^{14}C (sp act 250 μCi/mmol) in a final vol of 0.5 ml. Incubation was carried out for 10 min at 30° and terminated by addition of 50 μl of 6 N HCl. The acidified mixture was extracted 2× with 5 ml Et_2O and the combined Et_2O extracts transferred to scintillation vials and taken to dryness in a stream of N_2. Residue was taken up in 10 ml of blended toluene phosphor mixture (30% EtOH in toluene containing 4 g PPO per l.) and counted by standard liquid scintillation techniques. In some expts the Et_2O soluble fraction was separated by PC using the organic phase of toluene–HOAc–H_2O (10:7:3) and 95% of the Et_2O soluble radioactivity was found in the spot on the chromatogram corresponding to ferulic acid. Further proof of the nature of the product was obtained using two further solvent systems and specific colour reactions. In

all cases, the radioactivity corresponded with ferulic acid. No evidence for labelling of *iso*ferulic acid was obtained. Incorporation of leucine-^{14}C into a TCA insoluble fraction was followed using the method of ref. [22]. The protein concentration in the extracts was determined by the method of ref. [23] after precipitating aliquots of the extracts with 20% TCA and dissolving the resulting ppt. in 0.1 N NaOH.

Acknowledgements—One of the authors (A.C.R.H.) wishes to thank the Agricultural Research Council for the award of a postgraduate studentship.

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