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THE PROPERTIES OF CINNAMIC ACID 4-HYDROXYLASE OF AGED SWEDE ROOT DISKS

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Key Word Index—*Brassica napo-brassica*; Cruciferae; swede root; cinnamic acid 4-hydroxylase.

Abstract—The cinnamic acid 4-hydroxylase of swede root disks is a microsomal enzyme with a specific requirement for NADPH and cinnamic acid. The enzyme is inhibited under anaerobic conditions and by CO and this inhibition is partially reversed by light. The enzyme is insensitive to inhibitors of mitochondrial electron transport and to iron chelating agents. It is less sensitive to sulphydryl reagents and to product inhibition than the enzymes isolated from other plant sources. It is strongly inhibited by FAD and FMN at concentrations above 0·1 mM. NADH alone supports only very low rates of CAH activity but it has a synergistic effect in stimulating activity at limiting levels of NADPH. It has very little effect at NADPH levels approaching saturation and does not affect the V_{max} of the overall reaction. The particulate enzyme has been "solubilised" by treatment of the microsomal pellet with Triton X-100 with some variable loss of activity. The properties of the swede cinnamic acid hydroxylase are discussed in relation to other microsomal hydroxylation systems in plants and animals.

INTRODUCTION

The enzyme, cinnamic acid 4-hydroxylase (CAH) catalyses the first hydroxylation step in the pathway of phenolic biosynthesis [1], namely the conversion of cinnamic acid to *p*-coumaric acid. This enzyme is specific for hydroxylation in the *para* position and differs from the enzyme specific for *ortho*-hydroxylation recently described [2]. Two cinnamic acid 4-hydroxylases have been described in the literature. One isolated from spinach by Nair and Vining [3] has a pH optimum of 4·6 and a requirement for tetrahydrofolic acid and either NADPH or NADH for activity. The other enzyme isolated by Russell and Conn [4] and studied in detail by Russell [5] is particle bound with a specific requirement for NADPH and a pH-optimum of 7·5.

In previous studies it has been shown that ageing of disks of swede roots in the presence of ethylene leads to the formation of a lignin-like substance in the walls of the surface cells of the disks [6]. It was shown that during the process there was a marked increase in the activity of a number of enzymes involved in cinnamic acid and lignin biosynthesis [7–9]. In the present paper the properties of a cinnamic acid 4-hydroxylase in aged swede root disks will be discussed.

RESULTS

The cinnamic acid 4-hydroxylase activity of aged swede root disks was concentrated in the fraction of tissue homogenates which sediments between 10,000 and 80,000 × *g*. 95% of the total activity of the homogenate was recovered in the 80000 × *g* pellet which, when examined by electron microscopy, was shown to be composed principally of small vesicular bodies 40–400 nm in diameter and appears to correspond with the microsomal fraction of other plant tissues [10]. The contamination of this fraction by mitochondria was negligible but since the fraction was

* The work reported here is taken from the Ph.D. thesis submitted to the University of East Anglia (1975) by A.C. Hill.

Table 1. Effect of various additions to the CAH assay

Addition to assay	Concentration (mM)	Relative activity
None	—	100
Mercaptoethanol	0	75
	1	100
	2	100
	10	70
Iodoacetamide	1	70
p-Chloromercuribenzoate	1	75
p-Coumaric acid	5	90
Caffeic acid	5	49
Ferulic acid	5	100
Sinapic acid	5	102
Coniferyl alcohol	5	85
Potassium cyanide	1	140
FMN	0·01	100
	0·1	72
	1·0	8
FAD	0·01	106
	0·1	68
	1·0	9
KCl	100	100
	200	90
	500	45
	1000	11

orange in colour some of the vesicular material may have arisen from disintegration of chromoplast membranes during homogenisation.

The enzyme requires NADPH for activity; NADH will support only a limited degree of hydroxylation. It is inhibited under anaerobic conditions and has an apparent K_m for its substrate cinnamic acid of $2{\cdot}9 \times 10^{-5}$ M. The enzyme is fairly specific for cinnamic acid and shows no activity towards *p*-coumaric or ferulic acids. The pH optimum for activity is 7·5 (pH's for half maximal activities at pH 7·0 and pH 8·5) and is almost inactive at pH's below 6·0. The enzyme is relatively stable and microsomes in the presence of 1 mM DTE can be stored on ice for periods of up to 48 hr without significant loss of hydroxylase activity.

Table 1 shows the effect of various additions to the standard hydroxylase assay. The addition of mercaptoethanol at a concentration of 1–2 mM slightly stimulates the hydroxylation although higher concentrations were slightly inhibitory but the effect is not as great as that observed by Russell [5]. However, in the present experiments mercaptoethanol was included in the extraction medium as well as in the assay. Sulphydryl agents such as iodoacetamide and *p*-chloromercuribenzoate at 1 mM inhibit the enzyme by 25–30% but inhibitors of mitochondrial electron transport such as rotenone, antimycin A and 2,4-dinitrophenol have no effect on the hydroxylase. Similarly, metal chelating agents such as α,α'-dipyridyl are not inhibitory but potassium cyanide at 1 mM causes a marked stimulation of hydroxylation. Ferulic and sinapic acids (5 mM) have very little effect on CAH activity while coniferyl alcohol and *p*-coumaric acids cause only a small degree of inhibition. Caffeic acid at 5 mM, however, causes a 50% inhibition of CAH activity. The flavine nucleotides, FAD and FMN, both show strong inhibition of the swede CAH activity and at 1 mM inhibition with each nucleotide reaches 90%. The enzyme activity is also sensitive to high salt concentrations; concentrations above 0·2 M KCl are inhibitory and inhibition reaches 90% at 1·0 M KCl.

Table 2 shows the effect of carbon monoxide in causing inhibition of CAH activity. 5% CO in air causes 68% inhibition and this rises to 84% at 20% CO in air. This Table also shows that the inhibitory effect of CO is partially reversed by light even though light has no effect on the activity in the absence of CO.

The cofactor specificity of the swede microsomal CAH was investigated. The observed apparent K_m values (0·15 mM for NADPH and 7·0 mM for NADH) and maximum velocities (79·2 pKatals/mg protein for NADPH and 13·9 pKatals/mg protein for NADH) indicate a high degree of specificity of the system for NADPH. However, it was shown that NADH had a marked stimulatory effect on hydroxylation in the presence of limiting concentrations of NADPH. This effect is illustrated in Fig. 1. At 50 μM NADPH, 50 μM NADH causes a doubling of the rate of hydroxylation while at 500 μM NADPH this level of

Table 2. Inhibition of CAH by CO and its reversibility by light

Experiment No.		CAH activity pKat/mg protein	% inhibition
1	AIR control	52·8	
	AIR + 5% CO dark	16·7	68
	AIR + 10% CO dark	12·5	76
	AIR + 20% CO dark	8·3	84
2	AIR control light	72·2	
	AIR control dark	72·2	
	AIR + 5% CO light	45·8	37
	AIR + 5% CO dark	31·0	57

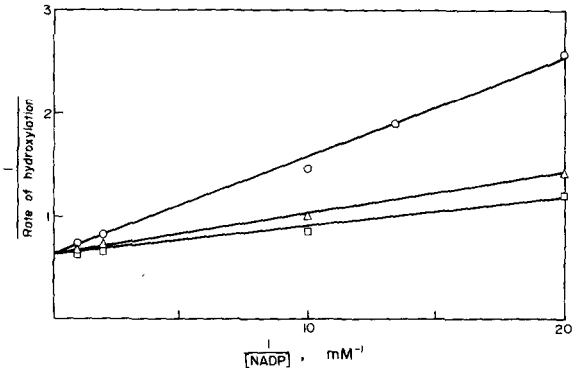

Fig. 1. A double reciprocal plot of cinnamic acid hydroxylase activity against NADPH concentration at different concentrations of NADH. ⊙ No NADH, △ 50 μM NADH, ⊡ 500 μM NADH.

NADH only causes a 12% increase in CAH activity. 50 μM NADH alone catalyses only a barely measureable rate of enzyme activity. Figure 1 shows the data from another experiment plotted by the method of Lineweaver and Burk [11]. It can be seen that NADH has a marked effect on the activity at low NADPH concentrations but has very little effect on the maximum velocity of the overall reaction.

Table 3 shows that $NADP^+$ at concentrations from 0·05–0·2 mM inhibits NADH-supported hydroxylation while it has only a small effect on NADPH-supported hydroxylation. NAD^+ (1 mM) has no effect on NADPH-supported CAH activity. The inhibition by $NADP^+$ of the NADH-supported CAH strongly suggests that a transhydrogenase activity cannot explain the role of NADH in the system.

The treatment of swede microsomal particles with the non-ionic detergent Triton X-100 leads to a somewhat variable loss of CAH activity (Table 4). In the most favourable preparations, very little loss of activity was found at Triton X-100 concentrations below 0·1% (see 4A). Above 0·1%, however, the system becomes unstable and activity is rapidly lost. Even at 0·1% the recoveries of activity are quite variable within the range of 40–100% retention of activity (Table 4B). However, when the Triton dispersed microsomes were re-sedimented at 80000 *g* for 2 hr, nearly 90% of the activity was found in the supernatant after centrifugation. A further study of the stability of CAH activity after dispersion of the microsomes with Triton X-100 shows that the major loss of activity occurs during the initial stages of Triton treatment. In one particular experiment there was a 23% loss of activity in the first hr after addition of Triton X-100, a further 25% loss in the next 24 hr and very little change in a further 24 hr period. A comparison of Triton X-100 and Triton N 101 [12] in their abilities to limit the initial loss of activity and subsequently to stabilise the

Table 3. Effect of oxidised pyridine nucleotides on CAH activity

	Addition to assay	Concentration of additive (mM)	CAH activity pKat/mg protein
1. Activity with NADH (1 mM) as cofactor	$NADP^+$	0	8
		0·05	4·8
		0·1	4·5
		0·2	2·2
2. Activity with NADPH (1 mM) as cofactor	$NADP^+$	0	72·2
		1·0	51·4
	NAD^+	1·0	72·2

Table 4. Effect of Triton X-100 on microsomal CAH activity

Experiment	Triton X-100 concentration (%)	CAH activity pKat/mg protein
A.	0	122·2
	0·05	113·8
	0·1	111
	0·5	13·8
	1·0	2·7
B. Microsomal pellet		94·4
Microsomal pellet after treatment with 0·1% Triton X-100		
	Before centrifugation	38·5
	After centrifugation	
	Pellet	2·7
	Supernatant	25·0

Table 5. The effect on the CAH activity of swede roots of washing the microsomal fraction

	CAH Activity* pKat/ml enzyme
Microsomal fraction	183
Microsomal fraction after washing	78
Supernatant from washing	16
Microsomal fraction after washing + supernatant from washing	144

* The values of activity given are the means of 4 assays carried out at four different enzyme levels. In the assays in which different fractions were mixed, levels of fractions equivalent to the same fr. wt of original tissue were included in the combined assays. 1 ml of enzyme was equivalent to 7 g fr. wt of tissue.

system showed that Triton X-100 was markedly superior to Triton N 101.

In an attempt to purify the microsomal fraction, the 80000 × *g* pellet was resuspended in buffer and re-centrifuged at 80000 × *g* for 2 hr to give a washed pellet and the washings. It was found in a number of experiments that this procedure led to a major loss in CAH activity. This is shown in Table 5 in which the CAH activities of various amounts of initial microsomal pellet, the washed pellet and the washings are compared. It can be seen that the CAH activity in the washings is very low but if the wash fraction is added back to the washed microsomes the CAH activity is almost completely restored to the level in the initial microsomal fraction. This suggests that at least one component of the CAH system is only loosely bound to the microsomal fraction.

DISCUSSION

The cinnamic acid 4-hydroxylase of swede roots has the same general properties as the enzyme described in pea seedlings by Russell and Conn [4,5] and no evidence for an enzyme of the type described by Nair and Vining [3] has been found in swede tissue. The swede enzyme is microsomal, requires O_2 and NADPH and is inhibited by CO and this inhibition is partially reversed by light. The enzyme is insensitive to inhibitors of mitochondrial electron transport and iron chelating agents such as α,α'-dipyridyl. The stimulatory action of cyanide on CAH activity is in agreement with Potts *et al.* [13]. The swede enzyme is, however, much less sensitive to inhibition by sulphydryl agents such as iodacetamide and *p*-chloromercuribenzoate and to product-inhibition by *p*-coumarate than are the enzymes from pea [5] and sorghum [13]. The inhibitory action of FMN and FAD is contrary to the findings of Vance *et al.* [14] in Polyporus in which FAD at 0·33 mM stimulated CAH activity. Microsomal CAH has now been demonstrated in a number of plant tissues [15–20] but its properties have only been studied in relatively few cases [5,13,18]. The present work conforms with the suggestion of Russell that plant microsomal CAH is a mixed function oxidase which is analogous in its behaviour to inhibitors to the microsomal hydroxylation systems of animal cells which have been shown to involve the cytochrome P450 in the hydroxylation reaction. The inhibition by CO and its photo-reversibility is a characteristic of this system and the findings of Potts *et al.* [13] that the action spectrum for photo-reversibility of the CO inhibition of sorghum CAH has a maximum at 450 nm strongly suggests that cytochrome P450 is involved in CAH activity. The involvement of an NIH shift [21] and of lipid components [20] in plant CAH activity provides further evidence of the similarity between CAH activity in plants and the animal microsomal hydroxylation system.

The animal hydroxylation system has been resolved into three major components, an NADPH specific flavoprotein reductase, cytochrome P450 and a lipid component [22]. The components of the animal system have been "solubilised" from the microsomal system by detergent treatments and the components resolved by chromatography [23]. The system can be reconstituted when the three components are re-mixed in the presence of NADPH, O_2 and substrate [23]. In addition, an NADH flavoprotein reductase and cytochrome b_5 [22] may be involved in hydroxylation but their role is unclear. It is a characteristic of the animal system that although NADH is not very active alone it acts synergistically in the presence of NADPH to support hydroxylation and it is thought that the NADH cytochrome *c* reductase and cytochrome b_5 may play a role in the synergistic effect of NADH [22].

The present work has shown that a similar synergistic effect of NADH at limiting NADPH levels on CAH activity occurs in the plant system and provides further evidence of the similarity of the animal and plant systems. In the swede system NADH has a relatively small effect at high NADPH concentrations and has little effect on the V_{max} of the overall reactions. In contrast, in the animal system NADH will stimulate hydroxylation markedly even at saturating NADPH levels [22]. The loss of activity on washing swede microsomes is further evidence that a multicomponent system is involved in CAH activity. The "solubilisation" of the plant CAH activity with Triton X-100 is, to our knowledge, the first time this has been achieved and the relative stability of the dispersed activity should enable us to study

in more detail the hypothesis [5] that cytochrome P450 and flavoproteins are involved in CAH activity. Further evidence on this point will be given in subsequent papers in this series.

EXPERIMENTAL

Disks (10 × 2 mm) of swede plant roots (*Brassica napo-brassica*) were prepared and aged under the conditions previously described [7]. 100 g aged disks were homogenised in a medium containing 0·1 M KH_2PO_4–KOH, 0·5 M sucrose, 1 mM EDTA, 2 mM merceptoethanol pH 7·5 in a stainless steel roller mill. The homogenate was filtered through Miracloth and centrifuged at 10000 *g* for 20 min. The supernatant after centrifugation was decanted and re-centrifuged at 80000 *g* for 120 min to obtain a microsomal pellet. The supernatant was decanted and the 2 pellet fractions re-suspended in a medium containing 0·1 M KH_2PO_4, 0·25 M sucrose, 0·5 mM EDTA, 2 mM mercaptoethanol pH 7·5. The microsomal fraction was used for most expts and CAH activity was measured by the method of Russell. Incubation included microsomal suspension, 0·1 M KH_2PO_4, 2 mM merceptoethanol, 0·5 mM cinnamic acid 2-^{14}C (4×10^5 dpm/μmol) and a NADPH regenerating system consisting of 1·6 mM $NADP^+$, 4·0 mM G-6-P and 1·75 units of G-6-P DH in a final volume of 1 ml. The NADPH regenerating system was incubated for 5 min at 25° prior to addition to the assay. The complete assay mixture was incubated at 25° for a further 10 min. and the reaction terminated by the addition of 0·1 mm 6 N HCl and 0·5 μmol of unlabelled *p*-coumaric acid to act as a carrier. The acidified reaction mixture was 2× extracted with 5 ml Et_2O and the combined Et_2O extracts taken down to dryness in N_2. Resulting residue was taken up in a minimum vol EtOH and applied to Whatman No. 20 paper. The *p*-coumaric acid was separated from any unreacted cinnamic acid by PC using the organic phase of toluene–HOAc–H_2O (10:7:3). The *p*-coumaric acid spot was located in UV light and after elution with 0·1 N NaOH its radioactivity determined by liquid scintillation counting. Suitable blanks using assays containing boiled enzyme were used to correct for any tailing of the cinnamic spot in the region of the *p*-coumarate. The reaction is linear with time for only a period of 15 min so in most experiments incubation periods of 5 or 10 min were employed. In separate experiments in which carrier *p*-coumarate was not added, the product was shown to be *p*-coumarate by its R_f in 3 solvents, its UV absorption after elution from the paper and its colour reactions with *p*-diazotised dianisidine. In 24 hr PC runs using the above system in which *p*-, *o*- and *m*-coumarate are resolved it was shown that the reaction product was the *p* isomer rather than the *o* or *m*-isomers. In experiments in which the composition of the atmosphere in which hydroxylation was carried out was changed, the reactions were carried out in Thunberg tubes which were flushed with gas mixtures of known composition made up in the laboratory from cylinders of the pure gases. In the light reversal experiments the reactions were run in a constant temperature bath and the Thunberg tubes were either covered with black cloth or illuminated with a 400 W fluorescent tube 40–50 cm from the tubes. In the expts in which the kinetics for individual cofactors were studied either separately or as mixtures, the NADPH regenerating system was replaced by the pure reduced cofactor at the concentrations stated in the text. In the attempts to solubilise the CAH activity the microsomal pellet was resuspended in a medium containing 25% glycerol, 0·05 M KH_2PO_4, 0·25 M sucrose, 0·5 mM EDTA and 1 mM dithiothreitol pH 7·5. Aliquots of Triton X-100 were added to the resuspended microsomes to give a final concentration of up to 1·0% and were analysed within 60 min or after centrifugation at 80000 *g* for 2 hr. In washing expts, the initial microsomal pellet was resuspended in 15 ml of resuspension medium and 12 ml then re-centrifuged at 80000 × *g* for 120 min. The resulting washed pellet was decanted from the washings and was resuspended in the same vol of resuspension medium. Washed microsomes and washings were assayed separately and together at a range of concentrations for CAH activity.

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