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REDUCTION OF THE CoA THIOESTERS OF *p*-COUMARIC AND FERULIC ACIDS BY EXTRACTS OF AGED *BRASSICA NAPO-BRASSICA* ROOT TISSUE

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Abstract—An enzyme system isolated from swede root disks, which had been aged in the presence of ethylene, is capable of the reduction of *p*-coumaric and ferulic acids to their corresponding aldehydes and alcohols in the presence of CoA, ATP, Mg^{2+} and a reduced pyridine nucleotide The reduction of *p*-coumaric acid specifically required $NADPH_2$ while that of ferulic acid is catalysed by either $NADPH_2$ or $NADH_2$ The involvement of the CoA thioesters as intermediates in the reduction of the cinnamic acids is discussed

INTRODUCTION

IN PREVIOUS papers[1-3] we have shown that there is an increased synthesis of a lignin like material during the ageing of swede root disks and that this is associated with an increase in the activity of enzymes involved in phenolic biosynthesis Recently we have described the properties of an enzyme which will catalyse the formation of CoA thioesters of cinnamic acid derivatives [4] The substrate specificity and changes in the activity of the enzyme during ageing suggest a role for the CoA synthetase enzyme in lignin biosynthesis CoA thioesters of cinnamic acids have been postulated as intermediates in flavonoid biosynthesis[5] and it has also been proposed that they play a role in the reduction of cinnamic acids to their corresponding alcohols which are the immediate precursors of lignin [6] The conversion of cinnamic acids to the alcohols proceeds via an aldehyde intermediate with the conversion of the aldehyde to the alcohol being catalysed by an aromatic alcohol dehydrogenase [7] The step from the acid to the aldehyde is thermodynamically unfavourable and it is proposed that this step is overcome by activation of the acid to form the CoA thioester and its reduction to the aldehyde Very recently[8,9] enzymes have been isolated from higher plant tissues which will catalyse the reduction of cinnamic acid derivatives to the alcohols via the CoA thioesters as intermediates In the present paper we de-

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[3] RHODES, M J C and WOOLTORTON L S C (1973) *Qual Plant Mat Veg* **23,** 145
[4] RHODES, M J C and WOOLTORTON, L S C (1973) *Phytochemistry* **12,** 2381
[5] HAHLBROOK, K , EBEL, J , ORTMANN, R , SUTTER, A , WELLMAN, E and GRISEBACH, H (1971) *Biochim Biophys Acta* **244,** 7
[6] ZENK, M H and GROSS, G G (1972) *Recent Adv Phytochem* **4,** 87
[7] DAVIES, D D , UGOCHUKWU, E N , PATIL, K D and TOWERS, G H N (1973) *Phytochemistry* **12,** 531
[8] MANSELL, R L , STOCKIGT, J and ZENK, M H (1972) *Z Pflanzenphysiol* **68,** 286
[9] EBEL, J and GRISEBACH, H (1973) *FEBS Letters* **30,** 141

scribe the properties of an enzyme isolated from aged swede root disks which will catalyse the reduction of *p*-coumaric and ferulic acids

RESULTS AND DISCUSSION

Table 1 shows the co-factor requirements of the enzyme system from aged disks in the reduction of *p*-coumaric acid to *p*-coumaryl alcohol The reduction requires a heat labile factor and the addition of ATP, CoA and $NADPH_2$ (or an $NADPH_2$ regenerating system composed of a mixture of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) The system is stimulated by the addition of Mg^{2+}, the rate in the absence of added Mg^{2+} is only 14% of the control The specificity of the reducing system in relation to CoA and ATP and the partial dependence on added Mg^{2+} is very similar to that of the *p*-coumaryl CoA synthetase previously described[4] and suggests a role for this enzyme in the reduction

TABLE 1 THE PROPERTIES OF THE ENZYME SYSTEM FROM AGED SWEDE ROOT DISKS REDUCING *p*-COUMARATE

	nmol *p*-coumaryl alcohol formed/hr/mg protein		nmol *p*-coumaryl alcohol formed hr/mg protein
Complete system	95	$-Mg^{2+}$	13
+ boiled enzyme	0	$-NADPH_2$ regenerating system	0
− ATP	0	Replace regenerating system	
− CoA	0	with 5 μmol $NADPH_2$	49

Table 2 shows the specificity with respect to reduced pyridine nucleotide of the reducing systems using respectively *p*-coumarate and ferulate as substrate With *p*-coumarate as substrate the enzyme shows complete specificity for $NADPH_2$ Both *p*-coumaryl alcohol and *p*-coumaryl aldehyde are formed as products of the reduction but the ratio of alcohol/aldehyde formed is about 15 1 With ferulate as substrate, the specificity is less clear and both $NADPH_2$ and $NADH_2$ will catalyse reduction However, $NADPH_2$ is 2 5 times as active as $NADH_2$ With $NADH_2$ as cofactor there is a proportionately greater accumulation of aldehyde than with $NADPH_2$ suggesting that the aromatic alcohol dehydrogenase step has a greater affinity towards $NADPH_2$ than towards $NADH_2$[10] At the concentration of substrate used (0 5 mM), the reducing enzyme shows greater overall activity towards ferulate than towards coumarate The ratio of activities is about 1 2 1

TABLE 2 THE REQUIREMENT OF THE ENZYME SYSTEM FROM AGED SWEDE ROOT DISKS FOR PYRIDINE NUCLEOTIDES IN THE REDUCTION OF *p*-COUMARIC AND FERULIC ACIDS

Experiment No 1	nmol formed/hr/mg protein Aldehyde	Alcohol	Experiment No 2	nmol formed hr/mg protein Aldehyde	Alcohol
(a) *p*-Coumarate as substrate			(a) *p*-Coumarate as substrate		
$NADH_2$	0	0	$NADH_2$	0	0
$NADPH_2$	1 2	17 3	$NADPH_2$	1 4	21 2
(b) Ferulate as substrate			(b) Ferulate as substrate		
$NADH_2$	4 0	5 8	$NADH_2$	2 0	8 4
$NADPH_2$	1 6	19 2	$NADPH_2$	1 3	28 2

[10] HAHLBROOK K and GRISEBACH H (1970) *FEBS Letters* **11**, 62

Table 3 shows the effect on the reducing system of factors known to affect the activity of *p*-coumaryl and feruloyl CoA synthetases We have shown, in agreement with the work of Grisebach,[9,10] that as the *p*-coumaryl CoA synthetase was purified, increased activity of the enzyme was observed indicating the removal of a soluble inhibitor present in crude extracts We have shown (unpublished results) that the addition of relatively high concentrations of Bovine serum albumin (BSA) will protect the enzyme in crude extracts from this inhibition The stimulatory effect of addition of BSA can reach up to 3-fold with crude fractions after desalting on Sephadex G25 columns but is reduced as the enzyme is successively purified Table 3 shows that the addition of BSA leads to a 20% stimulation of the overall reducing activity, which was measured after purification of the enzyme on DEAE-cellulose This degree of stimulation is similar to that given by BSA on the *p*-coumaryl CoA synthetase purified to this stage AMP, which as was previously shown[4] is a potent inhibitor of the *p*-coumaryl CoA synthetase, inhibits the reduction of *p*-coumarate by 74% and the reduction of ferulate by 63% It was previously shown that AMP at 2 5 mM gave a 69 5% inhibition of the *p*-coumaryl CoA synthetase [4]

TABLE 3 FACTORS AFFECTING THE ACTIVITY OF THE REDUCING SYSTEM

Cofactor or inhibitor	nmol of alcohol formed/hr/mg protein Substrate *p*-Coumarate	Ferulate
$NADPH_2$ + BSA (1 mg/ml)	24 9	28 2
$NADPH_2$ − BSA	20 5	23 7
$NADPH_2$ + BSA + AMP (2 5 mM)	6 6	10 4

The enzyme isolated from swede root disks after ageing is similar to the enzymes recently isolated from the cambial tissue of *Salix alba*[8] and tissue cultures of soya bean [9] The requirements for ATP, CoA and Mg^{2+}, the stimulation by BSA and the inhibition by AMP are consistent with a role for the *p*-coumaryl and feruloyl CoA synthetase activities as components of the reductase activities described in the present paper The swede reducing system shows an interesting difference in the co-factor specificity when *p*-coumarate and ferulate act as substrates *p*-Coumarate shows complete specificity for $NADPH_2$ while ferulate will use either $NADPH_2$ or $NADH_2$ This study, taken together with the work of Mansell *et al* [8] and Ebel and Grisebach,[9] confirms the role of the thioester of cinnamic acids as intermediates in the reduction of the acids to their corresponding aldehydes and alcohols

EXPERIMENTAL

The materials used and the methods of purification of the enzyme fraction after $(NH_4)_2SO_4$ precipitation, desalting on Sephadex G25 and purification on a column of DEAE-cellulose were as previously described [4]

Aliquots of the enzyme preparations were incubated in the following mixture Tris buffer pH 7 45 500 μmol, CoA 1 μmol, ATP 12 5 μmol, Mg^{2+} 12 5 μmol, BSA (Armour Pharmaceutical Co , Eastbourne, England) 5 mg, substrate acid 2 5 μmol, $NADPH_2$ regenerating system consisting of NADP 5 μmol, glucose-6-phosphate 12 5 μmol, glucose-6-phosphate dehydrogenase 1 EU (or $NADPH_2$ 5 μmol) in a final vol of 5 ml at 30° for up to 1 hr After this period the reaction pH was adjusted to 8 5 and the alcohol and aldehyde products extracted with Et_2O Aliquots of the Et_2O fraction were applied to Whatman No 20 filter papers and chromatograms developed with toluene–HOAc–H_2O (10 7 3), the products were located under UV light and eluted from the paper with EtOH In some experiments in which 2-^{14}C *p*-coumarate (sp act 60 μCi/mmol) was supplied as substrate, the ethanolic eluates of the reaction products were counted in the Philips liquid scintillation system using a blended scintillation mixture consisting of 7 parts toluene containing 4% PPO (2,5-diphenyloxazole) and

3 parts of the ethanolic eluate In other experiments in which unlabelled substrates were used the ethanolic eluates were analysed by UV spectrophotometry The concentrations of the aldehydes and alcohols were calculated from the absorbance data using the extinction coefficients given in the literature [11]

The main reaction products with *p*-coumarate and ferulate as substrates were identified by PC in toluene HOAc H_2O (10 7 3), *n*-BuOH-2N NH_4OH (1 1) and petrol (b p 100-120)–MeOH–$CHCl_3$-H_2O (7 1 2 5) The products respectively had the same R_fs and co-chromatographed with synthetic samples of *p*-coumaryl and coniferyl alcohols The products gave the colour reactions with diazotized dianisidine characteristic of the appropriate alcohol and its parent acid The neutral and alkaline UV spectra of the products after elution from the paper were identical with those of the respective synthetic alcohols Samples of the products purified by PC on solvent washed papers were introduced via the direct insertion probe to the source of the A E I Ltd MS 902 mass spectrometer at a source temp of 170 and an ionising potential of 70 eV The MS of the synthetic *p*-coumaryl and coniferyl alcohols were used to confirm the presence of these two compounds in the reaction mixtures containing respectively *p*-coumaric acid and ferulic acid as substrate The MS will be deposited at the Mass Spectrometry Data Centre Aldermaston, England

The presence of the respective aldehydes in the reaction mixtures was confirmed by the presence of compounds which had the same R_fs in the 3 solvents as *p*-coumaryl and coniferyl aldehydes and which stained with the phloroglucinol-HCl reagent On elution from the paper these aldehyde products gave UV spectra in neutral and alkaline solutions which agreed with literature values [11]

Protein determinations were made on TCA precipitates of the enzyme fractions using the method of Lowry *et al* [12]

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