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# ENZYMES INVOLVED IN THE REDUCTION OF FERULIC ACID TO CONIFERYL ALCOHOL DURING THE AGING OF DISKS OF SWEDE ROOT TISSUE

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**Key Word Index**—*Brassica napo-brassica*; Cruciferae; swede root; hydroxycinnamic acid: CoA ligase; ferulyl CoA ligase; aromatic alcohol dehydrogenase.

**Abstract**—The enzymes involved in the reduction of ferulic acid to coniferyl alcohol, namely hydroxycinnamic acid CoA ligase, ferulyl CoA reductase and an aromatic alcohol dehydrogenase, have been resolved by gradient elution on columns of DEAE-cellulose. The ferulyl CoA reductase has been separated into two fractions which are both specific for $NADPH_2$ and have a high affinity for their substrate. The aromatic ADH was resolved into two fractions, the second of which shows partial separation into two subfractions in extracts of aged but not of initial disks. The two ADH fractions are both specific for NADP but the second fraction has markedly higher affinity for substrate than the first fraction. In comparing the activities isolated from initial and aged disks, it is clear that both forms of ferulyl CoA reductase are present in initial disks and the increase on aging is only two-fold. The activities of ADH 1 and 2 do not alter during aging but the qualitative composition of Fraction 2 appears to alter. The hydroxycinnamic acid CoA ligase, however, is present only in very low concentration in initial disks and increases very markedly during aging. It is suggested that the control of the activity of this reduction system during the aging of swede root disks is mainly centred on the level of activity of the ligase enzyme. In addition, a method for the preparation and purification of ferulyl CoA, the substrate for the reductase enzyme, is described.

## INTRODUCTION

During aging, disks of the root of *Brassica napo-brassica* have been shown to form a layer of a lignin-like substance on their cut surfaces [1, 2]. An essential step in the biosynthesis of lignin is the reduction of hydroxycinnamic acids to their corresponding alcohols which are the immediate precursors of lignin [3]. In a previous paper [4], the enzymic reduction of *p*-coumaric and ferulic acids to *p*-coumaryl and coniferyl alcohols respectively was demonstrated in extracts of aged disks of swede roots and evidence was presented suggesting that the CoA thioesters of the acids [5] were intermediates in the reduction.

The enzyme system of swede root disks involved in the reduction of hydroxycinnamic acids is very similar to the enzymes described recently in cambial tissue of *Salix alba* [6], lignifying tissue of *Forsythia* [7-9] and cell suspension cultures of soyabean [10]. Gross *et al.* [8] have shown that three enzymes are involved, namely a hydroxycinnamic acid–CoA ligase (*p*-coumaryl CoA synthetase) [5, 9], a hydroxycinnamyl CoA reductase and an aromatic alcohol dehydrogenase. The present paper describes the partial purification and complete resolution of these three activities by chromatography on columns of DEAE-cellulose and some of the properties of the isolated enzymes. A comparison of the enzymic complement of initial and aged disks will be presented together with a method for the preparation of ferulyl CoA.

## RESULTS

The enzymes involved in the reduction of ferulic acid to coniferyl alcohol were separated on a column of Sephadex G-200. The separation of

enzymes on G-200 was far from complete but it showed the simultaneous elution of the *p*-coumaryl and ferulyl CoA ligase activities and the resolution of the NADP specific aromatic alcohol dehydrogenase (ADH) into two forms. The aromatic ADH's differ in elution position from an ethanol–NAD oxidoreductase which elutes between the two aromatic ADH peaks. The ferulyl CoA reductase elutes slightly later than the ligase activity and close to the second peak of the aromatic ADH. There is some suggestion of separation of two forms of the reductase.

Figure 1 shows the pattern of elution of the desalted 55–75% $(NH_4)_2SO_4$ fraction on DEAE-cellulose (DE32) using a linear gradient between 0·005 M Tris pH 7·45 and 0·4 M KCl in 0·005 M Tris pH 7·45 both containing 1 mM DTE. Complete resolution of the three activities was achieved. Aromatic ADH was separated into two peaks, 1 and 2, the second of which shows partial separation of two further components (2a and 2b). The two ligase activities were eluted simultaneously. The ferulyl CoA reductase was separated into two peaks A and B, with roughly equal activities in each. The reductase assay in fractions prior to chromatography on DEAE-cellulose is complicated by the presence of an active thioesterase. The reductase peaks after DEAE-cellulose chromatography are essentially free of thioesterase which was eluted with a peak at around Fraction 10 of the gradient elution.

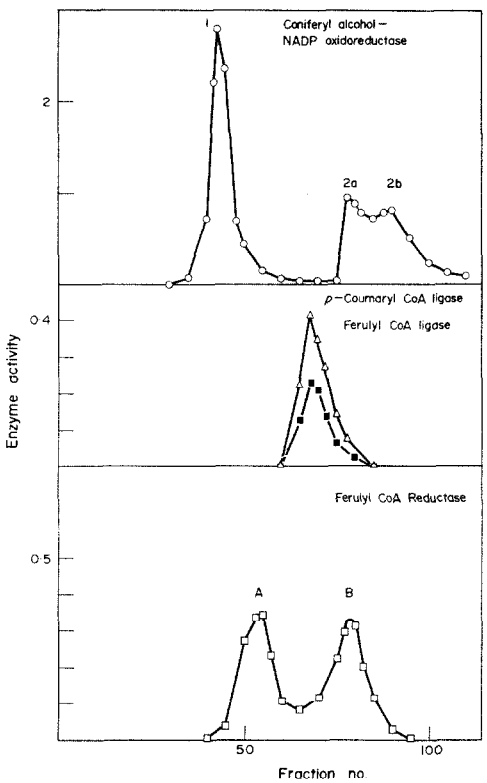


Fig. 1. The separation of enzyme activities from extracts of swede root disks aged in the presence of ethylene on a DEAE-cellulose column. △ *p*-Coumaryl CoA ligase; ■ ferulyl CoA ligase; ○ coniferyl alcohol:NADP oxidoreductase (aromatic alcohol dehydrogenase); □ ferulyl CoA reductase. Enzyme unit defined as the amount of enzyme to give a change in absorbance of 0·001/min under the defined assay conditions.

When the specific activities (activity/mg protein) of fractions at the peaks of activity of the enzymes after DEAE-cellulose chromatography were compared with the specific activities of the enzymes in the crude homogenate, degrees of purification of 51·7-fold for the ligase, 33·3-fold for aromatic ADH 1, 23·4-fold for aromatic ADH 2, 56-fold for ferulyl CoA reductase A and 42-fold for ferulyl CoA reductase B were obtained.

Table 1 shows the properties of the aromatic ADH's of swede root disks. These aromatic ADH's differ in substrate specificity, cofactor requirement and in elution position from the ethanol dehydrogenase, which elutes in a position near to that of the ligase in the DEAE-cellulose chromatographic separation (see Fig. 1). The aromatic ADH activity is much more stable than the ethanol dehydrogenase which is almost completely lost within 48 hr of the column elutions. Peaks 1 and 2 differ markedly in their affinities for coniferyl alcohol and aldehyde. There is a 30-fold difference in their $K_m$ values for coniferyl aldehyde and Peak 2 has a very high affinity for both coniferyl aldehyde and alcohol. Peaks 1 and 2 differ markedly in their susceptibility to substrate inhibition, the high affinity form being much more sensitive. Both fractions show high specificity for $NADP^+$ as the cofactor. No major differences in the kinetic properties of the subfractions a and b of Peak 2 have yet been observed.

Table 2 shows the properties of the ferulyl CoA reductase of swede root disks. The enzyme shows a marked specificity for $NADPH_2$ as the cofactor for reduction; NADH will sustain only 6% of the rate given by an equimolar concentration of $NADPH_2$. $Mg^{2+}$ will stimulate the activity but the effect is less marked than in the case of the

Table 1. The properties of the various forms of aromatic ADH of swede root tissue

| | Substrate | $K_m$ (μM) 1 | 2a | 2b |
|---|---|---|---|---|
| Aged disks | Coniferyl alcohol | 550 | NM | 2 |
| | Coniferyl aldehyde | 140 | 4 | 2 |
| Initial disks | Coniferyl aldehyde | 100 | — | 3 |
| Substrate inhibition | | No substrate inhibition at 75 μM coniferyl aldehyde or 750 μM coniferyl alcohol | | Strong substrate inhibition at 50 μM coniferyl aldehyde or at 60 μM coniferyl alcohol |
| Cofactor specificity (% NADPH dependent rate) | | | | |
| Coniferyl | NADPH | 100 | 100 | 100 |
| aldehyde | NADH | 2·6 | NM | 0·8 |
| Coniferyl | $NADP^+$ | 100 | 100 | 100 |
| alcohol | $NAD^+$ | ~0 | 0·73 | 0·86 |

NM = not measured; — = not present.

Table 2. The properties of ferulyl CoA reductases of swede root disks

| | (nmol/min/mg protein) | |
|---|---|---|
| Complete system | 21·4 | |
| $-Mg^{++}$ | 16·8 | |
| Replace $NADPH_2$ by $NADH_2$ | 1·3 | |
| | $K_m$ (μM) for ferulyl CoA | |
| | Peak A | Peak B |
| Initial disks | 5 | 7 |
| Aged disks | 4 | 3 |

Complete system given in Experimental.

Table 3. A comparison of the enzymic composition of extracts of initial and aged disks of swede roots

| | (nmol transformed/min/mg protein) Initial | Aged + 7 ppm ethylene |
|---|---|---|
| A. Crude homogenate | | |
| *p*-Coumaryl CoA ligase | ~0 | 2·92 |
| Ferulyl CoA ligase | ~0 | 2·07 |
| Ferulyl CoA reductase | 0·39 | 0·92 |
| Aromatic ADH | | |
| (a) Coniferyl alcohol oxidation | 5·3 | 6·15 |
| B. Fractions purified by bulk eluates from DEAE-cellulose (DE 23) | | |
| *p*-Coumaryl CoA ligase | ~0 | 8·88 |
| Ferulyl CoA ligase | NM | NM |
| Ferulyl CoA reductase | 1·35 | 3·74 |
| Aromatic ADH | | |
| (a) Coniferyl alcohol oxidation | 8·1 | 9·95 |
| C. Fractions after gradient elution on DEAE-cellulose (DE 32) | (nmol/min/100 g fr. wt) | |
| *p*-Coumaryl CoA ligase | <8 | 178 |
| Ferulyl CoA ligase | <5 | 121 |
| Ferulyl CoA reductase | | |
| A | 55 | 119 |
| B | 81 | 125 |
| Aromatic ADH 1 | 442 | 571 |
| 2 | 535 | 493 |

NM = not measured.

ligase [5]. Both forms of the enzyme show very high affinities for the ferulyl CoA substrate but there are no significant differences in the $K_m$ values of the two forms when prepared from either initial or aged disks.

Table 3 shows the activity of the three enzymes and their various subfractions in extracts of initial and aged disks measured at three different stages of purification. In expt A, the assays were carried out using the clarified homogenate and the results are expressed per mg of protein. It can be seen that the activities of *p*-coumaryl and ferulyl CoA ligase in initial disks are very low and are not detectable in dilute crude extracts. A large activity of both ligases was observed in extracts of disks after aging. The ferulyl CoA reductase activity in initial disks was substantial and the increase on aging was only about two-fold. The aromatic ADH is present in high activity in initial disks and does not alter substantially during aging. When these activities were measured on a bulk eluted fraction from DEAE cellulose (DE23) [5] the same pattern of changes in activity was observed (see Table 3B).

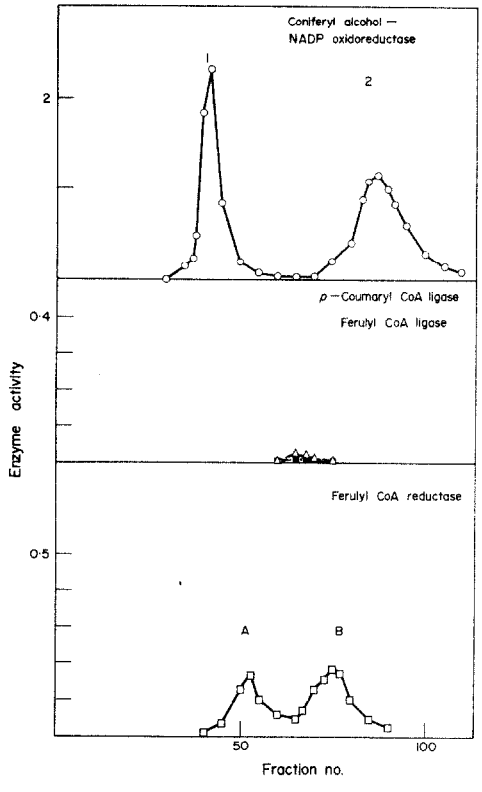


Fig. 2. The separation of enzyme activities from extracts of freshly prepared (initial) disks of swede roots on a DEAE-cellulose column. Symbols and conditions of elution exactly as in Fig. 1.

Figure 2 shows the separation of the enzyme activities in extracts from initial disks after gradient elution from DEAE-cellulose (DE32). Aromatic alcohol dehydrogenase is present in two forms which correspond in elution position (see Fig. 1) and in kinetic properties (see Table 1) to Peaks 1 and 2b of aged disks. The ligase enzyme is only found in trace amounts in initial disks. The ferulyl CoA reductase is present in two forms in initial disks and these correspond in elution position (compare Figs. 1 and 2) and kinetic properties (see Table 2) to the forms A and B described for aged disks. Table 3C shows the total activities present in the various forms of the enzymes of initial and aged disks. The two aromatic ADH's are present in roughly equal activities in both initial and aged disks and there is no change in total activity on aging. There is, however, the possibility that the qualitative composition of Peak 2 changes during aging. The two ferulyl CoA reductase enzymes are both present in equal activities in initial disks and there is a roughly two-fold increase in each fraction during aging. The ferulyl and *p*-coumaryl CoA ligases are present in extremely low levels in initial disks and there is an increase of at least 20-fold during aging.

## DISCUSSION

The three enzymes thought to be involved in the reduction of ferulic acid to coniferyl alcohol have been resolved by chromatography on DEAE-cellulose. Two of the enzymes show resolution into *iso*-enzymic forms but their significance is not entirely clear. The properties of the hydroxycinnamic acid—CoA ligase have been described in some detail in a previous publication [5] and this enzyme has similar properties to that of the enzyme isolated from *Forsythia* by Gross and Zenk [9]. The chromatographic studies, which show the simultaneous elution of the ligase activities with both ferulic and *p*-coumaric acids under a number of different conditions, suggest that a single protein is responsible for both ligase activities. The ferulyl CoA reductase shows separation into two forms which are both specific for $NADPH_2$ and which each has a very high affinity for the ferulyl CoA substrate. The spectrophotometric assay of Gross *et al.* [8]

has been used to localize the feruloyl CoA reductase activity. For the satisfactory measurement of activity by this method, it is essential that the aldehyde product of the reductase action is reduced to the alcohol. In relatively crude preparations, exogenous addition of aromatic ADH is not necessary but in fractions after gradient elution from the DEAE cellulose column, during which complete resolution of the reductase from the aromatic ADH was achieved, it was necessary to add back purified aromatic alcohol dehydrogenase in order to promote activity. The ADH Fraction 2 was much more efficient than Fraction 1 in promoting the reductase activity and this is correlated with the much higher affinity of Fraction 2 for coniferyl aldehyde (Table 1).

Davies *et al.* [11, 12] showed that potato tuber tissue contained three alcohol dehydrogenases: an aliphatic alcohol—NAD oxidoreductase; a terpene alcohol—NADP oxidoreductase; and an aromatic alcohol—NADP oxidoreductase. In the present work, we have shown the presence of two (or possibly three) aromatic alcohol:NADP oxidoreductases which are different from the aliphatic alcohol dehydrogenase. The two aromatic alcohol dehydrogenases, which are both specific for NADP, differ markedly in their affinities for substrate, and in their susceptibility to substrate inhibition. The aromatic ADH 2 has a $K_m$ value for coniferyl aldehyde of the order of $\mu$M and it seems likely that Fraction 2 is the enzyme involved in the reduction of coniferyl aldehyde to coniferyl alcohol.

A comparison of the enzymic complement of initial disks and disks aged in the presence of ethylene has shown that both the aromatic ADH and the feruloyl CoA reductase are present in substantial amounts in initial disks and that the changes in activity on aging are relatively small. Both major enzymic forms of the aromatic ADH are present in initial disks. Overall there is no difference in the total activity in Fraction 2 between initial and aged disks but the partial resolution of Fraction 2 into two subfractions (2a and 2b) in aged disks suggests that qualitative differences may occur within Fraction 2 during aging. The importance of this is not clear since both 2a and 2b have a high affinity for the aldehyde substrate. Both fractions (A and B) of the feruloyl CoA reductase are present in initial disks and these increase two fold in parallel during aging. In contrast to these results, the hydroxycinnamic acid:CoA ligase is present in only very low levels in initial disks and increases very markedly during aging. This result was confirmed when measurements of activity were made at various stages in a purification procedure known to remove the endogenous inhibitor of the enzyme found in swede root tissue [5]. Bovine serum albumin, which has been shown to counteract the action of the inhibitor [4], was included in all the assays of the ligase activity but did not stimulate activity in extracts of initial disks. These results strongly suggest that the presence of endogenous inhibitors is not an important factor in determining the observed activities. The data presented in this paper confirms the suggestion that three separate enzymes are involved in the reduction of hydroxycinnamic acids to hydroxycinnamyl alcohols and suggest that during aging of swede root disks the control of the system is at least in part through regulation of the level of activity of the enzyme, hydroxycinnamic acid–CoA ligase.

## EXPERIMENTAL

The preparation of disks of swede root tissue and the conditions for aging in the presence of 7 ppm ethylene are as previously described [13]. The extraction of enzymes and their purification by bulk elution from small columns of DEAE cellulose (Whatman DE23) and the assay procedure for the measurement of the hydroxycinnamic acid:CoA ligase are as previously described [5, 4].

*Extraction of enzymes and their purification by column chromatography.* 100 g of disks were homogenized in an Ultraturrax homogenizer for 1 min in 320 ml of a medium containing 0·1 M Tris, pH 8·0, 1 mM EDTA, 0·25 M sucrose, 1 mM dithioerythritol (DTE) and 1% polyclar AT. The homogenate was filtered through miracloth and clarified by centrifugation at 40000 *g* for 20 min. The supernatant was fractionated by $(NH_4)_2SO_4$ precipitation and the fraction between 55–75% satn $(NH_4)_2SO_4$ was desalted on a column of Sephadex G25 (medium grade, 2·5 × 35 cm), equilibrated with 0·005 M Tris pH 7·45 containing 1 mM DTE. The protein fraction from the G25 column was applied to a column of DEAE-cellulose (Whatman DE32) 1·5 × 10 cm equilibrated with 0·005 M Tris pH 7·45 containing 1 mM DTE. The fraction was washed on with the same buffer and the proteins eluted in a total vol. of 650 ml by a linear salt gradient between 0·005 M Tris pH 7·45 containing 1 mM DTE and 0·4 M KCl in the same buffer. 3 ml fractions were collected and assayed for the various enzyme activities. All procedures of the enzyme purification were carried out in a cold laboratory at 0–2°. During the various stages, samples of the protein fractions were taken, precipitated with 20% TCA and their protein contents determined by the method of Lowry *et al.* [14].

A similar procedure was used for the preparation of the enzyme fraction for purification on Sephadex G-200. The desalted fraction from G-25 was applied directly to a column of Sephadex G-200 (superfine grade, 2·5 × 36 cm) equilibrated

with 0·5 M KCl in 0·05 M Tris pH 7·45 and 1 mM DTE and eluted with the same buffer. 3 ml fractions were collected and assayed for enzyme activity.

*Assay of enzyme activities*: *Feruloyl CoA reductase*. (*a*) *Preparation of substrate*. The feruloyl CoA thioester substrate for the reductase assay was prepared by incubating purified swede root hydroxycinnamic acid:CoA ligase with 20 μmol ferulate, 8 μmol CoA, 100 μmol ATP and 100 μmol $MgCl_2$, 1 mmol Tris pH 7·45 in a final vol of 10 ml at 30° for 1 hr. Generally the bulk purified fraction from a column of DEAE cellulose (DE23) was used [5] but better yields were obtained if the peak of ligase activity after gradient elution from DE32 was used since this fraction is almost free of thioesterase activity. After incubation, the sample was cooled in ice, cold $Me_2CO$ added to a final concn of 80%, and made to acid pH with formic acid. The protein ppt was separated by centrifugation and discarded after washing with a further vol of 80% $Me_2CO$. The supernatant and washings were combined and evaporated under reduced pressure to remove the $Me_2CO$. The aq. residue was extracted eight times with ether to remove any unreacted ferulic acid. The aqueous phase was evaporated to dryness *in vacuo* and the residue taken up in 4 ml *n*-BuOH–HOAc–$H_2O$ (5:2:3) and applied to a column of Avicel microcrystalline cellulose (2·0 × 60 cm) equilibrated with *n*-BuOH–HOAc–$H_2O$. This same *n*-BuOH mixture was used to elute the CoA ester from the column. 2 ml fractions were collected and their UV absorption measured at 260 and 345 nm. Two peaks of absorption at 345 nm characteristic of the feruloyl CoA ester [15] were obtained (A and B) which ran ahead of the major 260 nm absorbing peaks due to ATP and CoA. Both peaks A and B had almost identical absorption spectra with well defined peaks at 257 and 345 nm [15]. Both show a characteristic initial bathochromic shift on addition of alkali followed by subsequent hydrolysis to yield ferulic acid. The purity of the samples was confirmed by PC and TLC in three solvents. In all the work in this paper the major component of feruloyl CoA thioester, B, has been used and component A discarded. Both A and B are active as substrates for the feruloyl CoA reductase to give coniferyl aldehyde but Fraction B is more active than A. The significance of the two peaks has not been studied in detail but it is possible that the resolution is into *cis* and *trans* isomers of the CoA esters.

(*b*) *Enzyme assay*. The method used is based on the spectrophotometric assay of Gross *et al.* [8]. A reaction mixture consisting of 0·1 M Tris pH 7·45 (100 μmol), $MgCl_2$ (2·5 μmol), DTE (100 μmol) and enzyme was incubated at 30° in both cells of a Unicam 1800 double beam spectrophotometer. 20 nmol feruloyl CoA were added to one cell, and the changes in absorbance at 345 nm followed to detect the presence of thioesterase activity. Then after about 3 min 0·24 μmol of $NADPH_2$ was added to each cell and the additional rate due to reductase activity followed for about 10 min. The rate of change of absorbance was linear over about 5 min. Thioesterase was a problem at all stages up to the gradient elution chromatography on the DEAE 32 when the reductase is almost completely separated from the thioesterase activity.

In fractions after gradient elution from the DEAE-cellulose column which are free of aromatic ADH, it is necessary to add back purified high affinity ADH (Fraction 2). This fraction which is free of reductase and thioesterase was added to ensure rapid conversion of coniferyl aldehyde to the alcohol. Chemical aldehyde trapping agents and commercially available horse liver ADH were tried in place of the plant aromatic ADH but proved much less satisfactory.

The enzyme unit is defined as the amount of enzyme giving a rate of change of absorbance at 345 nm of 0·001/min under the defined conditions. When measurements of total activity are given the activity is defined in terms of nmol of feruloyl CoA disappearing per min per mg protein using an EC of 31 000 $M^{-1}cm^{-1}$ and for overall conversion of feruloyl CoA to coniferyl alcohol.

*Aromatic alcohol dehydrogenase*. Aromatic alcohol dehydrogenase was assayed spectrophotometrically at 30° using $NADP(H_2)$ as cofactor and either coniferyl alcohol or coniferyl aldehyde as substrate. The assay system is as described by Gross *et al.* [8], except that in the direction of aldehyde reduction, pH 7·45 was used instead of 8·0.

*Ethanol dehydrogenase*. Ethanol dehydrogenase was measured as previously described [16].

## REFERENCES

1. Rhodes, M. J. C. and Wooltorton, L. S. C. (1973) *Phytochemistry* **12,** 107.
2. Rhodes, M. J. C. and Wooltorton, L. S. C. (1973) *Qual. Plant. Mat. Veg.* **23,** 145.
3. Freudenberg, K. and Neish, A. C. (1968) *Constitution and Biosynthesis of Lignin*, Springer, Berlin.
4. Rhodes, M. J. C. and Wooltorton, L. S. C. (1974) *Phytochemistry* **13,** 107.
5. Rhodes, M. J. C. and Wooltorton, L. S. C. (1973) *Phytochemistry* **12,** 2381.
6. Mansell, R. L., Stöckigt, J. and Zenk, M. H. (1972) *Z. Pflanzenphysiol.* **68,** 286.
7. Stöckigt, J., Mansell, R. L., Gross, G. G. and Zenk, M. H. (1973) *Z. Pflanzenphysiol.* **70,** 305.
8. Gross, G. G., Stöckigt, J., Mansell, R. L. and Zenk, M. H. (1973) *FEBS Letters* **31,** 283.
9. Gross, G. G. and Zenk, M. H. (1974) *European J. Biochem.* **42,** 453.
10. Ebel, J. and Grisebach, H. (1973) *FEBS Letters* **30,** 141.
11. Davies, D. D., Patil, K. D., Ugochukwu, E. N. and Towers, G. H. N. (1973) *Phytochemistry* **12,** 523.
12. Davies, D. D., Ugochukwu, E. N., Patil, K. D. and Towers, G. H. N. (1973) *Phytochemistry* **12,** 531.
13. Rhodes, M. J. C. and Wooltorton, L. S. C. (1971) *Phytochemistry* **10,** 1989.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
15. Gross, G. G. and Zenk, M. H. (1966) *Z. Naturforsch.* **21b,** 683.
16. Rhodes, M. J. C. (1973) *Phytochemistry* **12,** 307.