Pergamon

0031-9422(94)00753-5

Phytochemistry, Vol. 38, No. 3, pp. 573–577, 1995

0031-9422/95 $9.50 + 0.00

DITHIOTHREITOL AND COBALT EFFECTS ON MEMBRANE-ASSOCIATED PEROXIDASES OXIDIZING FERULOYL-CoA

KATHRYN E. MYTON* and STEPHEN C. FRY†

Division of Biological Sciences, The University of Edinburgh, Daniel Rutherford Building, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, U.K.

(*Received in revised form* 23 *August* 1994)

Key Word Index—*Zea mays*; Poaceae; maize; endo-membrane isolation; feruloyl-CoA metabolism; peroxidase; thiols; cobalt; feruloyltransferase.

Abstract—Membrane preparations from cell-suspension cultures of maize produced ^{14}C-polymeric material from [*methoxy*-^{14}C]feruloyl-CoA under conditions reported to be suitable for the assay of feruloyl-CoA:polysaccharide feruloyltransferase activity. The major ^{14}C-polymer formed did not contain *O*-[^{14}C]feruloyl-arabinofuranosyl groups. Production of ^{14}C-polymer was prevented by catalase, indicating a requirement for endogenous H_2O_2, and was Co^{2+}-dependent but only in the presence of dithiothreitol (DTT). The membranes exhibited peroxidase activity with guaiacol and H_2O_2; this reaction was blocked by DTT. It is concluded that peroxidases and H_2O_2, endogenous to the isolated membranes, caused oxidative polymerization of [^{14}C]feruloyl groups of feruloyl-CoA; DTT prevented polymerization by acting as a competitive substrate for peroxidases, and Co^{2+} restored polymerization by forming an insoluble complex with the thiol.

INTRODUCTION

The occurrence of peroxidases associated with membrane fractions of the cell has been reported in several species [1–4]. Generally they are thought to be only loosely bound to membranes although an integral membrane-bound peroxidase has been localized in the plasma membrane [5]. The enhanced binding of peroxidases to membranes in the presence of Ca^{2+} and Mn^{2+} and the removal of peroxidase activity from the microsomal fraction by treatment with concentrated salt solutions suggest that they are ionically associated with the membranes [3, 6]. Peroxidases are glycoproteins and inhibition of binding of both basic and anionic peroxidases to membranes by tunicamycin suggests that the carbohydrate moiety of the enzyme is involved in the binding process [2, 6].

Studies using differential centrifugation suggest that peroxidases are membrane-associated during their transport to the wall in secretory vesicles, whence they are released into the wall [3]. Anionic and cationic peroxidases have been found both associated with membranes and in the cell wall [7, 8] and it is likely that some of the peroxidases found associated with membranes were in the process of being transported.

Microsomally associated peroxidases with high affinities for hydroxycinnamic acids have been reported [4, 9]. The products formed when these peroxidases act on phenolic acids are often specific dimers (e.g. 8,8′-diferulic acid) suggesting they may be involved in the cross-linking of phenolic residues of the cell wall [10–18]. It has generally been assumed that the formation of diferulate and related oxidative phenolic coupling products occurs within the cell wall. However, it is possible that the microsomally associated peroxidases are also involved in phenylpropanoid metabolism within the endomembrane system.

The results reported in this paper describe the activity of a microsomally associated peroxidase capable of utilising feruloyl-CoA to form a polymeric product and the effects on this process of a thiol (dithiothreitol) and cobalt ions. This activity may be related to a putative feruloyltransferase responsible for the feruloylation of nascent polysaccharides [19, 20].

RESULTS

Maize membrane preparations were capable of incorporating radioactivity from [^{14}C]feruloyl-CoA, in the presence of DTT and Co^{2+}, into polymeric material in a time- and temperature-dependent manner. Evidence that the product was polymeric was its ability to bind to paper during copious water washing (Fig. 1) and its insolubility

*Present address: Centre de Biologie et Physiologie Végétales, Unité Associée au CNRS 1457, Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse Cédex, France.

†Author to whom correspondence should be addressed; fax + 44 131 650 5392.

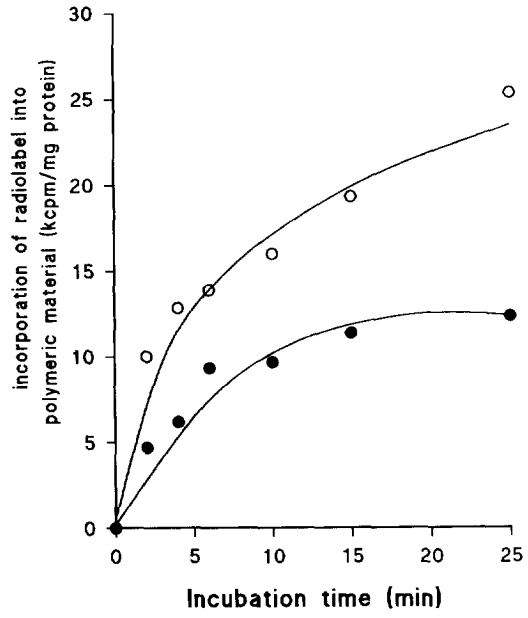

Fig. 1. Effect of arabinoxylan on the kinetics of incorporation of ^{14}C from feruloyl-CoA into polymeric material. Cell cultures were homogenized in the presence (○) or absence (●) of arabinoxylan. Assay mixtures contained 5 mM $CoCl_2$, 1 mM DTT, 0.5 kBq [*methoxy*-^{14}C]feruloyl-CoA, and maize endomembrane preparation. Each point represents the mean of three replicate assays from two membrane preparations.

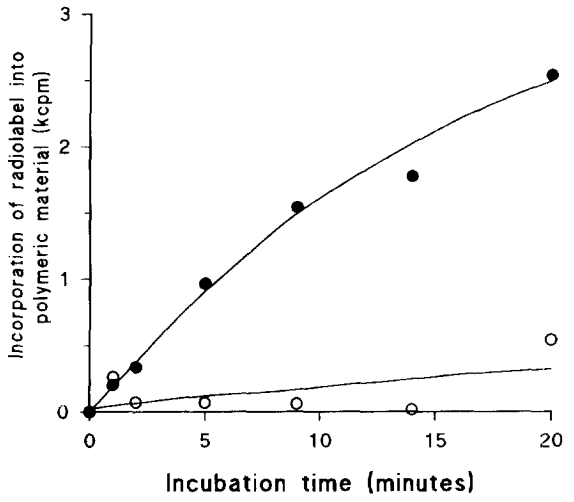

Fig. 2. Effect of catalase on the kinetics of incorporation of ^{14}C from feruloyl-CoA into polymeric material. Assay mixtures contained 0.5 kBq [*methoxy*-^{14}C]feruloyl-CoA, 1 mM DTT, 2 mM $CoCl_2$, and the membranes, plus either 55 units of catalase (○) or an equivalent volume of buffer (●). Each point is the mean of duplicate assays from two membrane preparations.

in ethanol–acetic acid (3:2). The addition of arabinoxylan during cell homogenization increased the rate and prolonged the duration of the reaction (Fig. 1). Boiling the membranes for 5 min destroyed this activity. However, the ^{14}C-polymeric material yielded no detectable 5-*O*-[^{14}C]feruloyl-arabinose on partial acid hydrolysis (data not shown). In contrast, feruloylated arabinose residues that occur as side-chains on xylan-based polysaccharides are readily liberated in the form of 5-*O*-feruloyl-arabinose upon hydrolysis in 0.1 M TFA at 100° for 1 hr [20]. These observations indicate that the radioactive polymer formed in the present experiments was not the major *O*-feruloyl-arabinoxylan found in maize cell walls [21].

Little incorporation of ^{14}C into polymeric material occurred unless Co^{2+} was present in the assay mixtures and the optimum concentration for this ion was ~ 5 mM; markedly less incorporation occurred at 7–10 mM (data not shown). Other divalent cations tested (Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}) were ineffective and the addition of EDTA to assays inhibited the formation of ^{14}C-polymer (data not shown).

Added catalase almost completely inhibited the reaction in the short term (0–15 min; Fig. 2) although small amounts of ^{14}C-polymer were formed after longer incubation of assays containing catalase (up to 1 hr; data not shown). The inhibition of the reaction by catalase suggests that the incorporation of ^{14}C from [^{14}C]feruloyl-CoA into polymeric material was H_2O_2-dependent and presumably due to the activity of one or more peroxidases present in membrane preparations.

The presence of peroxidase activity in the membrane preparations was confirmed by their ability to oxidize guaiacol in the presence of exogenous H_2O_2. Addition of 1 mM DTT to assays temporarily inhibited guaiacol oxidation (Fig. 3). Plant peroxidases have been shown to possess thiol oxidase activity [22] and ascorbate peroxidase has been shown to become inactivated by thiols, e.g. dithiothreitol, in the presence of H_2O_2 [23]. In the absence of DTT and $CoCl_2$ (but presumably in the presence of endogenous H_2O_2) membrane preparations incorporated ^{14}C from [^{14}C]feruloyl-CoA into polymeric material (Fig. 4). This activity was inhibited by the addition of 1 mM DTT to assays; however, 2 mM $CoCl_2$ reversed this inhibition and resulted in activity only slightly less than in assays lacking both DTT and $CoCl_2$ (Fig. 4).

DISCUSSION

Membranes isolated from maize cell cultures possessed peroxidase activity which was able to oxidize guaiacol and to incorporate ^{14}C from [^{14}C]feruloyl-CoA into polymeric products. This activity was catalase-sensitive although some slight production of ^{14}C-polymer occurred during prolonged incubation in the presence of catalase. This long-term reaction could be because the high K_m for H_2O_2 of catalase would result in incomplete scavenging by catalase. Alternatively, peroxide-independent side-reactions could occur during prolonged incubation.

DTT inhibited the oxidation of guaiacol and [^{14}C]feruloyl-CoA. This is likely to be because the thiol groups were preferentially oxidized [22] by the peroxidase in the membrane preparations and it was only after most of the thiol groups had been oxidized that the

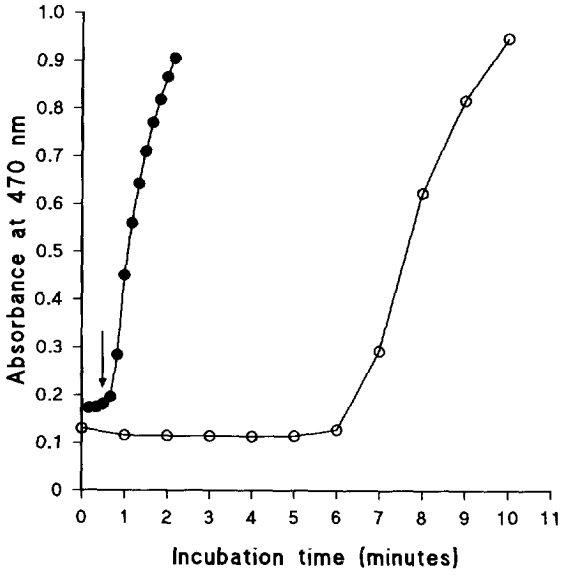

Fig. 3. Effect of DTT on the oxidation of guaiacol by membrane preparations. Assay mixtures contained 4 mM guaiacol and 2 mM H_2O_2 in phosphate buffer, pH 7.5, with the addition (○) or exclusion (●) of 1 mM DTT. No cobalt was added. The arrow indicates the time of addition of 100 μl membrane preparation (total vol. 1 ml).

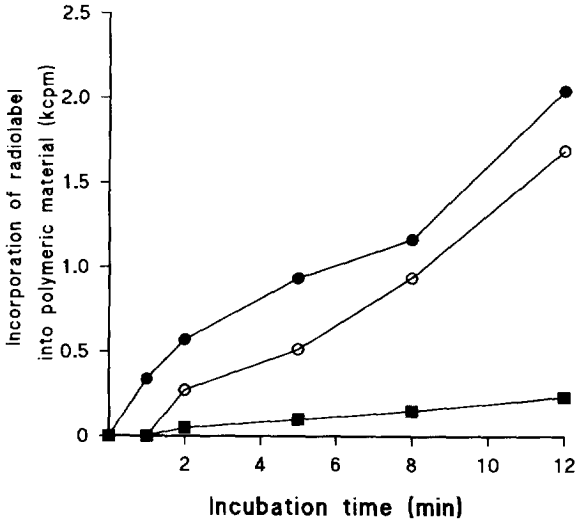

Fig. 4. Interactive effect of Co^{2+} and DTT on the incorporation of ^{14}C from feruloyl-CoA into polymeric material. Assay mixtures contained a maize membrane preparation, [^{14}C]feruloyl-CoA, and either (●) no additions, (■) 1 mM DTT, or (○) 1 mM DTT and 2 mM $CoCl_2$.

phenol was utilized as a substrate. In the presence of DTT, peroxidase activity against [^{14}C]feruloyl-CoA was only evident when Co^{2+} was present in the assay mixture and it is likely that this was not due to Co^{2+} acting as a co-factor or stabilizing ion but because of its interaction with DTT. Certain heavy metals interact with thiols, often forming precipitates. This is due to the production of slightly soluble metal hydroxides, which form insoluble conjugates with the thiol [24, 25]. Thus in the assays described here we suggest that cobalt reacts with the DTT present in the membrane preparations, removing the thiol from solution. In this way the DTT is no longer available as a substrate for the peroxidase and the [^{14}C]feruloyl-CoA will be utilized, misleadingly giving the impression that Co^{2+} is a specific co-factor in the formation of high-M_r products. The decline in activity seen at higher Co^{2+} concentrations could be due to inhibition of the peroxidase by the metal ion.

Incorporation of ^{14}C from feruloyl-CoA into insoluble material was stimulated and prolonged by the addition of arabinoxylan to the medium in which the cells were homogenized. It has been reported that some carbohydrates can enhance the oxidative coupling of phenols: for example, peroxidase-generated dehydrogenation products of ferulic acid can become linked, by alkali-stable bonds, to carboxymethylcellulose or filter paper [26–28], and dehydrogenation polymers of coniferyl alcohol become covalently linked to carbohydrates when present in the reaction medium [18, 29]; these reactions probably proceed via free radical and quinone methide intermediates [29–33]. It is likely that the products of peroxidase action on [^{14}C]feruloyl-CoA became linked to endogenous polysaccharides and glycoproteins or exogenous arabinoxylan present in the membrane preparations. Alternatively, the polysaccharide molecules could act as a framework which may non-covalently interact with the feruloyl-CoA, perhaps optimizing the orientation of the molecules for oxidative polymerization.

Although the presence of peroxidases in the microsomal fraction of the cell has been established [1–4], little is known about their role, if any, in the endo-membrane system. Peroxidases are likely to be transported, after production in the endoplasmic reticulum, whilst associated with membranes but it is also possible that they have a specific physiological role within the endo-membrane system. Peroxidases are thought to be responsible for the oxidative coupling of phenolic groups of the primary cell wall and this has generally been considered as a wall-located process. However, evidence suggests that the rate of formation of diferulate bridges is sometimes controlled not by the rate of oxidative coupling of feruloyl residues but by the rate of feruloylation of the polysaccharides [15, 17] since the ferulate:diferulate ratio remains constant regardless of the extensibility of the wall. Feruloylation occurs post- or co-synthetically to nascent polysaccharides [20, 34] and possibly dimerization of phenolic residues of these newly formed polysaccharides may occur before secretion into the wall, ensuring strict control of the ferulate:diferulate ratio.

Informative data on the roles of membrane-associated peroxidases may be gained through the use of immuno- and histochemical localization of the enzymes and their potential substrates; in addition, the determination of the substrate specificities of membrane-associated isoenzymes of peroxidase may prove useful in assessing their physiological role.

The problems arising from the presence of peroxidases in membrane fractions have been discussed [4] with respect to the isolation of plant cytochrome P450s and it is prudent to be aware of artifactual results that may arise owing to the presence of peroxidases in membrane preparations. This is particularly true when phenolic or other substrates of peroxidases are being utilized in assays for non-oxidative enzyme activities such as feruloyltranferases [19].

EXPERIMENTAL

Materials. [*methoxy*-^{14}C]Feruloyl-CoA (2.11 TBq mol^{-1}) was synthesized at Zeneca Agrochemicals, Jealotts Hill Research Station, Berks, U.K. by the method of ref. [35]. Arabinoxylan (from wheat flour) was from Megazyme, Australia. Catalase (from bovine liver) was from Sigma Chemical Co. Cell suspension cultures of maize (*Zea mays* L.) were maintained in Murishage and Skoog's basal salts mixture [36] supplemented with sucrose (20 g l^{-1}), MES (100 mg l^{-1}), 2,4-dichlorophenoxyacetic acid (2 mg l^{-1}), N^6-(2-isopentenyl)adenine (2 mg l^{-1}), α-naphthaleneacetic acid (0.5 mg l^{-1}), pyridoxine-HCl (0.5 mg l^{-1}) and thiamine (0.1 mg l^{-1}); final pH 5.8; cultures were shaken in dim light.

Isolation of endo-membranes. Maize cells (40 g, 4-day-old cultures) were rinsed in ice-cold 10 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) containing 1 mM dithiothreitol (DTT) (final pH adjusted to 7.2 with NaOH) and then disrupted in an ice-cold soln containing 50 mM MOPS, 1 mM DTT, 1 mM EDTA, 0.4 M sucrose and 0.5% bovine serum albumin (BSA) (pH adjusted to 7.2 with NaOH) by 5 min homogenization in a teflon-in-glass Potter type blender at 300 rpm in an ice-bath. The homogenate was filtered through 2 layers of muslin and centrifuged at 20 000 *g* and 4° for 15 min in order to pellet a thioesterase activity which otherwise hydrolysed feruloyl-CoA. The supernatant was then re-centrifuged at 100 000 *g* for 25 min. The resulting microsomal pellet was washed and resuspended in buffer as above but lacking EDTA and BSA and re-centrifuged at 100 000 *g* for 25 min. The re-pelleted microsomes were routinely resuspended in 50 mM MOPS, 1 mM DTT (pH 7.2) at 0° and used in enzyme assays on the day of preparation.

Assay of ^{14}C*-polymer formation.* Routinely, aliquots (75 μl) of membrane preparation plus any additives (final vol. 100 μl) were supplied with [*methoxy*-^{14}C]feruloyl-CoA (0.5 kBq, 2.4 μM), incubated at 18–22°, terminated by the addition of 10 μl HCO_2H and dried on Whatman 3MM filter paper (3 × 4 cm strips). The papers were washed in cold running water for 4 hr and incorporation of ^{14}C into polymeric material remaining adsorbed to the paper was monitored by scintillation counting of the re-dried paper strips by immersion in 2 ml 0.5% (w/v) 2,5-diphenyloxazole (PPO), 0.05% 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP) in toluene.

The effect of divalent cations on activity was investigated by supplying a range of concentrations (1–10 mM) of $CoCl_2$, $MnCl_2$, $MgCl_2$ or $ZnSO_4$ to individual assays; EDTA was added at a concentration of 1 mM to control assays. The H_2O_2-dependency of the activity was tested by the addition of either 55 μkat of catalase (1 μkat will decompose 1.0 μmol of H_2O_2 s^{-1} at 25° and pH 7.0 while the H_2O_2 concentration falls from 10.3 to 9.2 mM) or an equivalent vol. of buffer.

In order to investigate the possible participation of membrane-associated polysaccharides in the formation of [^{14}C]feruloyl-containing polymeric material, microsomal fractions were prepared through homogenization of cells in buffer to which arabinoxylan (10 mg ml^{-1}) had been added. During this process it is suggested that polysaccharide chains become encapsulated in membrane vesicles and are thus available for use as a substrate.

Guaiacol peroxidase activity was assayed by supplying 100 μl membrane suspension with 4 mM guaicol, 2 mM H_2O_2 in phosphate buffer pH 7.5 (total vol. 1 ml) and monitoring the increase in absorbance at 470 nm in the presence or absence of 1 mM DTT. Membranes were also assayed for their ability to utilize [^{14}C]feruloyl-CoA (i) with 1 mM DTT, (ii) with 1 mM DTT and 2 mM $CoCl_2$ and (iii) without either.

Analysis of ^{14}C*-labelled products.* Samples of the ^{14}C-polymeric products, collected after pptn in EtOH–HOAc (3:2), were treated with 0.1 M TFA at 100° for 1 hr—conditions shown to release 5-*O*-feruloyl-L-arabinose from maize cell walls [20]. Products were analysed by PC on Whatman 3 MM in BuOH–HOAc–H_2O (12:3:5), EtOAc–pyridine–H_2O (8:2:1), and EtOAc–HOAc–H_2O (10:5:6); authentic 5-*O*-feruloyl-L-[^{3}H]arabinose [20] was added as an internal marker. Strips of the chromatography paper were assayed for both ^{14}C and ^{3}H by liquid scintillation counting.

Acknowledgements—K.E.M. was funded by a studentship from the Science and Engineering Research Council in collaboration with Zeneca Agrochemicals. S.C.F. thanks the Agricultural and Food Research Council for a research grant.

REFERENCES

1. Penel, C. and Greppin, H. (1979) *Phytochemistry* **18**, 29.
2. Penel, C. (1991) in *Biochemical, Molecular and Physiological Aspects of Plant Peroxidases* (Lobarzewski, J., Greppin, H., Penel, C. and Gaspar, T., eds), pp. 225–236. University of Geneva, Geneva.
3. Ros Barceló, A. R., Muñoz, F. and Sabater, F. (1988) *Plant Physiol. Biochem.* **26**, 575.
4. Rodgers, M., Zimmerlin, A., Werck-Reichhart, D. and Bolwell, G. P. (1993) *Arch. Biochem. Biophys.* **304**, 74.
5. Askerlund, P., Larsson, C., Widell, S. and Moller, I. M. (1987) *Physiol. Plant.* **71**, 9.
6. Kiefer, S., Penel, C. and Greppin, H. (1985) *Plant Sci.* **39**, 37.
7. Zheng, Z. and van Huystee, R. B. (1992) *Plant Sci.* **81**, 47.
8. Ferrer, M. A., Pedreno, M. A., Ros Barceló, A. and

Muñoz, R. (1992) *J. Plant Physiol.* **139**, 611.
9. Gröger, W., Förster, U. and Pommer, U. (1993) in *Natural Phenols in Plant Resistance, Book of Abstracts*, p. 4. International Society of Horticultural Science, Munich.
10. Geissmann, T. and Neukom, H. (1971) *Helv. Chim. Acta* **54**, 1108.
11. Markwalder, H.-U. and Neukom, H. (1976) *Phytochemistry* **15**, 836.
12. Hartley, R. D. and Jones, E. C. (1976) *Phytochemistry* **15**, 1157.
13. Fry, S. C. (1979) *Planta* **146**, 343.
14. Fry, S. C. (1980) *Phytochemistry* **19**, 735.
15. Kamisaka, S., Takeda, S., Takahashi, K. and Shibata, K. (1990) *Physiol. Plant* **78**, 1.
16. Hartley, R. D. and Morrison, W. H. (1991) *J. Sci. Food Agric.* **55**, 365.
17. Tan, K.-S., Hoson, T., Masuda, Y. and Kamisaka, S. (1992) *Plant Cell Physiol.* **33**, 103.
18. Wallace, G. and Fry, S. C. (1994) *Int. Rev. Cytol.* **151**, 229.
19. Meyer, K., Köhler, A. and Kauss, H. (1991) *FEBS Letters* **290**, 209.
20. Myton, K. E. and Fry, S. C. (1994) *Planta* **193**, 326.
21. Kato, Y. and Nevins, D. J. (1985) *Carbohydr. Res.* **137**, 139.
22. Pichorner, H., Couperus, A., Korori, S. A. A. and Ebermann, R. (1992) *Phytochemistry* **31**, 3371.
23. Chen, G.-X. and Asada, K. (1992) *Plant Cell Physiol.* **33**, 117.
24. Hopton, J. D., Swan, C. J. and Trimm, D. L. (1968) *Adv. Chem. Ser.* **75**, 216.
25. Capozzi, G. and Modena, G. (1974) in *The Chemistry of the Thiol Group* (Patai, S., ed.), pp. 785–839. John Wiley & Sons, New York.
26. Stafford, H. A. (1964) *Plant Physiol.* **39**, 350.
27. Siegel, S. M. (1967) in *Comprehensive Biochemistry*, (Florkin, A. and Stotz, E. H., eds), Vol. 26A, pp. 1–51. Elsevier, Amsterdam.
28. Whitmore, F. W. (1976) *Phytochemistry* **15**, 375.
29. Ohnishi, J., Watanabe, T. and Koshijima, T. (1992) *Phytochemistry* **31**, 1185.
30. Freudenberg, K. and Harkin, J. M. (1960) *Chem. Ber.* **93**, 2814.
31. Leary, G. J. (1980) *Wood Sci. Technol.* **14**, 21.
32. Watanabe, T., Kaizu, S. and Koshijima, T. (1976) *Chem. Letters* 1871.
33. Takahashi, N. and Koshijima, T. (1988) *Wood Sci. Technol.* **22**, 231.
34. Fry, S. C. (1987) *Planta* **171**, 205.
35. Stöckigt, J. and Zenk, M. H. (1979) *Z. Naturforsch.* **30c**, 352.
36. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.