# Purification and partial characterization of a peroxidase from plant cell cultures of *Cassia didymobotrya* and biotransformation studies[1]

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An acidic peroxidase (EC 1.11.1.7) produced by cell suspension cultures of *Cassia didymobotrya* (wild senna) was purified from culture medium collected on the 29th day. The enzyme was shown to be a glycoprotein with a pI of 3.5, a molecular mass of approx. 43 kDa by SDS/PAGE and 50 kDa by gel filtration. The N-terminal sequence was very similar to those of other plant peroxidases. The peroxidase was characterized by a high speci-ficity towards coniferyl alcohol and other natural phenolics such as guaiacol and ferulic and caffeic acids. These findings suggest that the enzyme is involved in lignification processes of the cell wall. Moreover, the enzyme was able to catalyse the oxidation of 4,3′,4′-trihydroxychalcone and 4,3′,4′-trihydroxy-3-methoxy-chalcone to the corresponding 3,3′-biflavanones, as mixtures of racemic and *meso* forms.

## INTRODUCTION

Peroxidases are enzymes that are widely distributed in the plant kingdom and can be found in vacuoles, tonoplast, plasmalemma and inside and outside the cell wall. These enzymes have a variety of functions as evidenced by the presence of several isoenzymes in plant cell compartments. They are involved in plant hormone regulation [1], defence mechanisms [2–4], control of cell elon-gation, polymerization of extensin [5], cross-linkage of cell wall polysaccharides [6], lignin biosynthesis [7] and the suberization processes [8].

Plant tissue-culture studies provide a powerful tool to in-vestigate their functions and specificity. We have shown that in cell cultures of *Cassia didymobotrya* different peroxidase iso-enzymes are present that, according to their electrophoretic mobilities, can be divided into anionic and cationic isoforms [9]. The most acidic ones are confined to the cell wall or extracellular sites, whereas the cationic forms are distributed both in the cell wall and the cytoplasmic compartments.

We have also shown that crude cell-free extracts isolated from suspended cells of *C. didymobotrya* were able to catalyse the conversion of chalcones **1a** and **1b** to the corresponding flavones (**2a/2b**) and 3,3″-biflavanones (**3a/3b** or **4a/4b**) (Figure 1) [10]. In this paper we describe the purification procedure and initial studies on the physical and catalytic properties of an anionic isoenzyme.

## MATERIALS AND METHODS

### Materials

Enzyme preparations were obtained from stabilized and opti-mized suspension cultures of *C. didymobotrya* (wild senna) [10]. Because we observed a comparable reactivity towards 4,2′,4′-trihydroxy-3-methoxychalcone for cell-free extract and spent medium, we chose the medium as enzyme source because it was easier to handle. The synthesis of chalcones **1a** and **1b** was as reported previously [11]. All other chemicals were from Aldrich, Sigma and Bio-Rad.

### Enzyme assay

The activity of the enzyme during the purification was monitored by spectrophotometric measurements of the oxidation products of guaiacol at 470 nm. The reaction was performed at 30 °C in 3.1 ml of reaction mixture containing 25 mM sodium acetate buffer, pH 5.5, 0.25 mM $H_2O_2$, 0.3 mM substrate and suitable amounts of enzyme. One unit is defined as the amount of enzyme that catalysed the oxidation of 1 $\mu$mol of guaiacol per min per ml of enzyme preparation at 470 nm ($\epsilon_{470} = 26.6$ mM$^{-1} \cdot$cm$^{-1}$).

### Other peroxidase assays

The activity of acidic peroxidase and horseradish peroxidase (HRP) on various substrates was calculated by the decrease in the following absorption maxima: ascorbic acid (290 nm), NADH (340 nm), coniferyl alcohol (262 nm), ferulic acid and vanillin (310 nm) and caffeic and coumaric acids (285 nm), whereas for tyrosine and indole 3-acetic acid the calculation was based on the increase in absorption at 260 nm. All the substrates were at a final concentration of 0.3 mM, and NADH was at a final concentration of 0.03 mM; $H_2O_2$ was used at 0.2 mM. For all the assays a sodium acetate buffer (25 mM) at pH 5.5 was used. The two enzymes (acidic peroxidase and HRP) were employed at the same concentrations as calculated from their respective haem absorbances taking $\epsilon_{403}$ as 91 mM$^{-1} \cdot$cm$^{-1}$ for *C. didymobotrya* peroxidase and as 102 mM$^{-1} \cdot$cm$^{-1}$ for commercial HRP-C.

### Protein content

Protein concentration was determined both by the method of Bradford [12], with BSA as a standard, and by measurement of the absorbance at 280 nm.
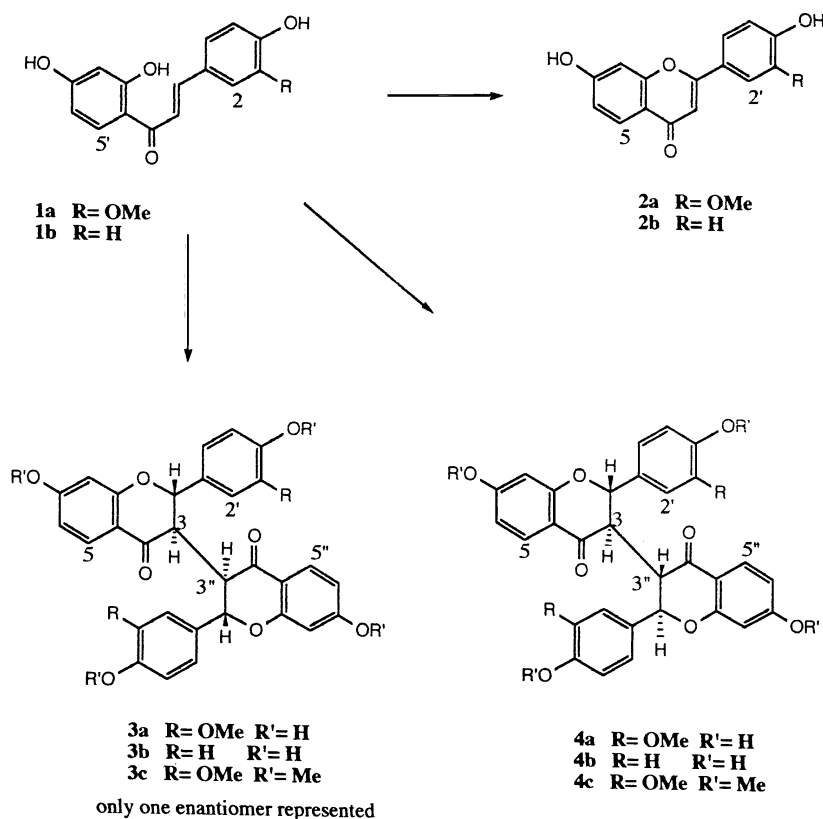
**Figure 1     Biotransformation of 4,2′,4′-trihydroxy-3-methoxychalcone**

Biotransformation of 4,2′,4′-hydroxy-3-methoxychalcone (**1a**) catalysed by the purified anionic peroxidase from suspension cultures of *C. didymobotrya* gave the corresponding 3,3′-biflavanones (**3a** and **4a**), but there was no evidence of the presence of compound (**2a**), which was instead recovered after treatment of compound (**1a**) with an unpurified enzymic mixture [10].

## Kinetic analysis

Both chalcone **1a** and coniferyl alcohol were dissolved in the minimum volume of methanol. Kinetic experiments were performed with a Cary 3E UV–Vis spectrophotometer at a constant temperature of 25 °C. The final anionic peroxidase concentration was 3 nM. The reactions were followed as a linear decrease in absorbance at 264 nm for coniferyl alcohol and 380 nm for chalcone in the first seconds of reaction. The initial rates and [$H_2O_2$] were fitted to an equation of the type [13]:

$$v/2[E]_0 = A[H_2O_2]/(B + [H_2O_2]) \qquad (1)$$

Parameters $A$ and $B$ were used to calculate the rate constants $k_1$ and $k_3$. In these experiments substrate concentrations ranged from 5 to 100 $\mu$M, whereas [$H_2O_2$] ranged from 50 to 200 $\mu$M.

## Thermal stability

Acidic peroxidase and HRP-C were each incubated in 1 ml of 25 mM sodium acetate buffer, pH 5.5, at 50 °C or 65 °C for 20 h. During this period five checks were done to monitor the stability of the two enzymes; 0.1 ml aliquots were taken and assayed for peroxidase activity with the guaiacol assay (see above).

## Biotransformations

Biotransformations of chalcones were performed in 50 mM acetate buffer, pH 5.5, at 30 °C. The reaction mixture contained, in a final volume of 5 ml, anionic peroxidase and HRP-C (0.02 mM final concentration), $H_2O_2$ (18 mM final concentration)

and the chalcone (0.35 mM final concentration). The reaction time was 10 min, after which the reaction was stopped with a suitable volume of ethyl acetate. The resulting organic phase was then dried under a stream of nitrogen or, for bigger volumes, by evaporation under vacuum.

## Biotransformation products

The biotransformation of 4,2′,4′-trihydroxychalcone and 4,2′,4′-trihydroxy-3-methoxychalcone by peroxidases was followed by TLC on silica gel in a chloroform/methanol (9:1, v/v) solvent system. The same system was used for preparative TLC. The products were analysed by mass spectroscopy and by ^{1}H and ^{13}C NMR spectroscopy (300 and 75 MHz respectively, in acetone-d$_6$ with tetramethylsilane as internal standard).

## Purification steps

All the steps described below were performed at 4 °C.

### Treatment with polyvinyl polypyrrolidone (PVPP)

The medium from 29-day-old cell suspension cultures of *C. didymobotrya* was collected after filtration of the cells and treated under gentle stirring overnight with PVPP at a PVPP-to-medium ratio of 1 g/10 ml.

## Filtration on Sephadex G-25

The medium was concentrated by ultrafiltration with an Amicon YM-10 membrane. The material obtained was loaded on a Sephadex G-25 (Pharmacia) column (16 cm × 2.5 cm) equilibrated with 25 mM sodium acetate buffer, pH 5, and the active colourless fractions were collected.

## First ion-exchange chromatography

Because of the large extent of medium-sized (14–34 kDa) basic peptides found in the medium, the sample was loaded on a CM Macro-Prep (Bio-Rad; 10 cm × 1.5 cm) column equilibrated with 25 mM sodium acetate buffer, pH 5, and the excluded fraction was collected as the 'acidic fraction'. The sample was finally equilibrated with a 25 mM Tris/HCl buffer at pH 7.5 by dialysis.

## Second ion-exchange chromatography

The 'acidic fraction' was loaded on a DEAE-Sepharose (Pharmacia) column (25 cm × 1.5 cm) equilibrated with 25 mM Tris/HCl buffer, pH 7.5, and eluted with a NaCl gradient from 0 to 0.2 M. The acidic peroxidase was released from the column at a NaCl concentration of 0.08 M.

## Gel filtration

The active fraction collected from DEAE-Sepharose was concentrated to 2 ml and then applied to a Sephacryl-S200 HR (Pharmacia) column (85 cm × 1 cm) equilibrated with 25 mM sodium acetate buffer/0.5 M NaCl/1 mM CaCl$_2$ (pH 7.4). The column was eluted with the same buffer at a flow rate of 0.5 ml/min, collecting fractions of 1 ml.

## Electrophoresis

The proteins were separated by native 7.5 % PAGE [14] and by SDS/PAGE (10 % gel) [15]. Isoelectric focusing was performed on polyacrylamide tube gels, with an Ampholine range between 3 and 10 and subsequently between 3 and 5. The gels were stained for peroxidase activity with guaiacol in sodium acetate buffer, pH 5, and for total protein content with Coomassie Brilliant Blue R-250. Periodate/Schiff staining was used to detect glycosidic residues.

### Molecular mass

The molecular mass of the peroxidase was estimated by SDS/PAGE with the High Range Molecular Weight Standard (36 000–205 000) from Sigma and by gel filtration under the same conditions as described above.

### N-terminal amino acid sequencing

After SDS/PAGE, the band at 43 kDa corresponding to *C. didymobotrya* peroxidase was electrotransferred to PVDF membrane (ProBlott; Applied Biosystems) by the method of Matsudaira [16]. Sequence analysis was performed with an Applied Biosystems Model 473A pulsed liquid sequencer with an online phenylthiohydantoin amino acids analyser.

### Other studies

Treatments of peroxidase with $\alpha$-mannosidase was performed as described previously [17].

## RESULTS AND DISCUSSION

### Production of crude acidic peroxidase

The activity of acidic peroxidase in the medium was measured at different ages of the culture. The major enzymic activity was obtained from 29-day-old cultures (Figure 2), corresponding to the end of exponential phase of the growth curve.

### Purification

The purification procedure is summarized in Table 1. The first steps of the purification scheme, through PVPP and Sephadex G-25, were performed to eliminate the phenolics that were present in the culture medium. A certain amount of pigments that remained in the samples could be separated from the proteins only with Sephacryl S-200 HR equilibrated with a buffer containing 0.5 M NaCl. The final absorption ratio between $A_{403}$ and $A_{280}$ ($R_Z$: purity index) of 2.8 was a good indication of a purified haem protein; moreover, N-terminal analyses did not yield other contaminant signals.

### Molecular mass, pI and purity determination

SDS and native electrophoresis of the purified peroxidase showed a single band when stained with guaiacol or Coomassie Blue R-
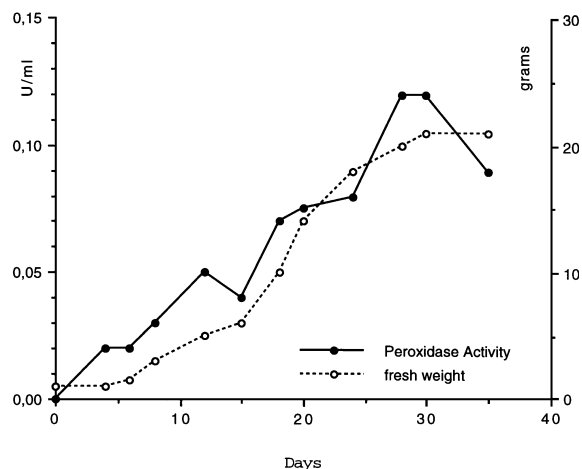
**Figure 2   Expression of purified peroxidase in the spent medium during aging of cells**

The peroxidase activity was calculated for samples (5 ml) of the medium obtained from a rapid purification by Sephadex G-25 and cation-exchange columns. The enzyme assay was the same as that reported in the Materials and methods section.

**Table 1   Purification of peroxidase from suspension cultures of *C. didymobotrya***

| Step | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Purification (fold) | Yield (%) |
|------|------|------|------|------|------|
| G25 | 104.7 | 14 | 7.45 | 1 | 100 |
| Macro Prep CM | 57 | 0.32 | 179.25 | 24 | 55 |
| DEAE-Sepharose | 22.2 | 0.02 | 1140.7 | 153 | 21 |
| S200-HR | 16.5 | 0.011 | 1525 | 204 | 16 |

**Table 2    Comparison between amino acid sequences of *C. didymobotrya* and other peroxidases**

| Source | Type | Sequence |
|---|---|---|
| *C. didymobotrya* | Anionic peroxidase | **D Y N T S X P N A A S T I K D G V** |
| *Brassica rapa* | P7 cationic peroxidase | F **Y S T S** C **P N** L L **S T** V **K S G V** |
| *Arachis hypogaea* | Cationic peroxidase | F **Y** A **T** K C **P N A L S T I K S** A **V** |
| *Oryza sativa* | Peroxidase precursor | F **Y** D **T S** C **P N A L S T I K S** A **V** |
| *Hordeum vulgaris* | Peroxidase precursor | F **Y** D **T S** C **P** R A L A **T I K S G V** |

**Table 3 Spectroscopic properties of purified peroxidase from *C. didmymobotrya***

| Derivative | Absorption maxima (nm) | $\epsilon$ (mM$^{-1}\cdot$cm$^{-1}$) |
|---|---|---|
| Native form | 403 | 91.2 |
| | 510 | 24 |
| | 632 | 18 |
| Compound I | 410 | 75 |
| | 524 | 7.5 |
| | 555 | 6.1 |
| Compound II | 417 | 68.7 |
| | 550 | 7.8 |
| | 530 | 7.6 |
| Compound III | 414 | 71.7 |
| | 577 | 5.3 |
| | 547 | 3 |
| Reduced form | 433 | 72 |
| | 555 | 21.4 |
| Pyridine haemochromogen | 419 | 90 |
| | 554 | 34.4 |

250. The presence of other isoenzymes was excluded after isoelectric focusing experiments in which the same samples appeared as one narrow coloured band when stained by both methods.

After heating at 60 °C and after separation on an SDS gel system, the enzyme maintained its ability to oxidize guaiacol and to develop brown bands on the gel. Treatment with 2-mercaptoethanol did not yield additional bands, indicating that the enzyme consisted of a single polypeptide chain. The molecular mass calculated by electrophoresis was 43 kDa, whereas by gel filtration the enzyme was eluted in a volume corresponding to a molecular mass of approx. 50 kDa.

When coloured in an SDS gel system, a broadening of the enzyme band over a range of 6 kDa could be observed. This and the increased molecular mass indicated by gel filtration can be attributed to the high glycosylation of the protein. The observed pI of the peroxidase after analytical isoelectric focusing was 3.5.

### Structural characterization

The peroxidase, blotted on a hydrophobic membrane as described in the Materials and methods section, was subjected to microsequencing analysis without any treatment to remove the linked carbohydrates. The N-terminal amino acid analysis gave structural information corresponding to the following residues: Ala[1]-Arg-Phe-Asp-Tyr-Asn-Thr-Ser-Xaa-Pro-Asn-Ala-Ala-Ser-Thr-Ile-Lys-Asp-Gly-Val[20]. This sequence, comprising the first 20 residues of the mature *C. didymobotrya* peroxidase, shows a high degree of similarity to the corresponding regions of plant peroxidases and plant precursor peroxidases (Table 2).

### Spectroscopic properties

The native enzyme showed absorption maxima at 403, 510 and 632 nm. The data obtained from the enzyme's pyridine haemochromogen identified the haem as an iron protoporphyrin IX. The absorption spectra of some enzyme derivatives were characteristic of a plant peroxidase (Table 3) [18–22]. Compound I was obtained by treatment of the purified enzyme with equimolar $H_2O_2$. The spectrum of compound II was observed after addition of an equimolar amount of potassium ferricyanide. An excess (3 mM) of $H_2O_2$ produced the spectrum of compound III. In addition, a reduced form of the enzyme was obtained with a small amount of sodium dithionite.

### Carbohydrate composition

The electrophoretic behaviour of the peroxidase, in both SDS/PAGE and native PAGE, suggested that the peroxidase was a glycoprotein, as confirmed by periodate/Schiff staining. Plant peroxidases mostly have oligosaccharide chains linked to asparagine, containing mannose and glucosamine residues with mannose elements more exposed to the environment. To attempt a partial characterization of the sugar composition of the enzyme, the purified peroxidase was applied to a concanavalin A column with a loading 0.1 M sodium acetate buffer, pH 6.0, containing 1 M NaCl, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. Only a small amount of protein was eluted initially; the majority was released with an $\alpha$-D-mannose solution (1 M). The strong affinity of the enzyme for concanavalin A can be explained by the presence of mannose chains on the surface of the enzyme. The fraction bound to the lectin column was therefore treated with $\alpha$-mannosidase, resulting in a loss of molecular mass (approx. 2 kDa), whereas enzyme activity was completely retained. These findings strongly suggest that mannose is not essential for peroxidase stability.

### Effect of pH and inhibitors

The peroxidase has an optimum pH of 5.5 with guaiacol and coniferyl alcohol as substrates. The buffers used (all at 50 mM) were: glycine/HCl (pH 3), sodium acetate (pH 4 and 5), phosphate, (pH 6 and 7), Tris/HCl (pH 8) and glycine/NaOH (pH 9). The enzyme was completely inhibited by cyanide.

### Enzyme localization and specificity

The acidic peroxidase was mainly found in the spent medium and, in a smaller amount, in the water washings of the whole cells. When the cells were washed with 0.2 M $CaCl_2$, the band attributed to acidic peroxidase did not appear after native electrophoresis but at least two cationic isoenzymes were found. This diversity in distribution was related to a difference in the net charge of the isoenzymes [23]. In contrast, the literature dealing with peroxidases describes both anionic [24] and cationic [25,26]

**Table 4    Substrate specificity of purified peroxidase from *C. didymobotrya***

Various natural compounds were incubated with anionic *C. didymobotrya* peroxidase and with HRP. The highest activity of *C. didymobotrya* peroxidase was expressed towards coniferyl alcohol and ferulic acid. Both these compounds belong to the lignin formation pathway and, notably, bear a vanillin-type substitution on the aromatic ring like the chalcone (**1a**). No activity was expressed towards *p*-hydroxyphenols, in which *C. didymobotrya* peroxidase markedly differed from HRP. Activity is expressed as $\mu$mol of substrate transformed per min by 1 mg of enzyme.

| | Activity ($\mu$mol/min per mg) | |
|---|---|---|
| Substrate | *C. didymobotrya* peroxidase | HRP-C |
| Coniferyl alcohol | 303 | 306 |
| Ferulic acid | 232 | 257 |
| Caffeic acid | 120 | 240 |
| Coumaric acid | 0 | 282 |
| Guaiacol | 108 | 180 |
| Vanillin | 2.2 | 43 |
| Ascorbic acid | 108 | 168 |
| NADH | 3 | 4.2 |
| Tyrosine | 0 | 46 |
| IAA | 0 | 60 |
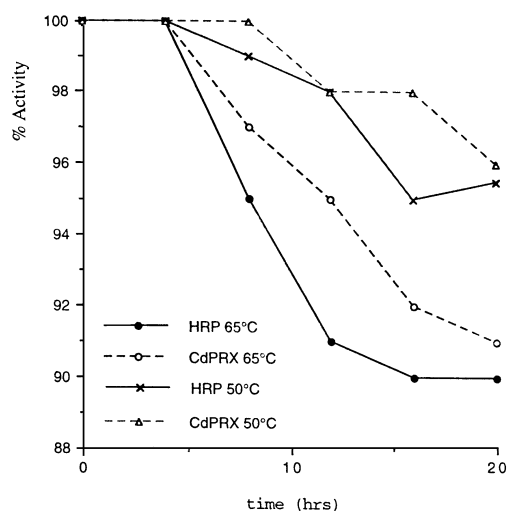


**Figure 3    Thermal stability of *Cassia* anionic peroxidase (CdPRX) and HRP**

The enzymes were left for 20 h in two baths at 50 °C and 65 °C. The stability of the two enzymes was almost completely retained. The checks were done at 4, 8, 12, 16 and 20 h by measuring the activity of 0.1 ml of enzyme preparation on guaiacol as described in the Materials and methods section. Activity is shown as percentage of the control experiment (at 25 °C) for clarity.

extracellular enzymes. Another feature that could influence the location of the enzyme is the interaction with certain ions, e.g. $Ca^{2+}$ ions, which are known to have an important role in the plant peroxidase's activity and secretion [27]. Preliminary experiments of elicitation with $CaCl_2$ solutions resulted in an increase in the excretion of the anionic peroxidase.

To attempt an identification of the physiological role of the purified peroxidase, a number of potential natural substrates were used. The compounds that were preferentially oxidized by the enzyme (see Table 4) belong to that class of phenols involved in the lignification of the cell wall [28]. A comparison with commercial HRP-C showed a difference in the specificity of the

two enzymes with respect to some substrates. It is of interest that acidic peroxidase does not recognize *p*-hydroxyphenols at all, in marked contrast with HRP, which is also able to oxidize indole 3-acetic acid.

The pH optimum, extracellular localization and specificity for phenolic compounds strongly suggest a role for this enzyme in the cell wall architecture and particularly in lignin biosynthesis. Other typical roles of peroxidases, such as hormone regulation via indole 3-acetic acid oxidation and $H_2O_2$ production, seem to be excluded.

### Thermal stability

After 20 h at 50 and 65 °C, both acidic peroxidase and HRP showed a minimal loss of activity, indicating that they are very stable enzymes in semicritical thermal situations (Figure 3). Preliminary experiments showed that the stability of acidic peroxidase was higher when $Ca^{2+}$ ions were present in the buffer.

### Kinetic analysis

The peroxidase from *C. didymobotrya* followed typical Michaelis–Menten kinetics when initial oxidation rates of substrates (coniferyl alcohol and chalcone **1a**) were plotted against reducing substrate concentration, [AH]. In accordance with the generally accepted mechanism for peroxidase reaction [29], the rates of disappearance of reducing substrates against [$H_2O_2$] were fitted for each concentration of substrate (5–100 $\mu$M), obtaining a series of rectangular hyperbolas. By using eqn. (1) to fit data points, parameters $A$ and $B$ were acquired and used to calculate $k_1$ and $k_3$ (means $\pm$ S.D.), which were respectively $(8.8 \pm 0.5) \times 10^7$ $M^{-1} \cdot s^{-1}$ and $(6.3 \pm 0.2) \times 10^7$ $M^{-1} \cdot s^{-1}$ for coniferyl alcohol, and $(5.5 \pm 0.2) \times 10^7$ $M^{-1} \cdot s^{-1}$ and $(5.0 \pm 0.1) \times 10^7$ $M^{-1} \cdot s^{-1}$ for chalcone **1a**. By plotting $A$ against $B$, straight lines with slopes $k_1$ (rate of disapperance of compound I) were obtained for both substrates, indicating that the *Cassia* enzyme follows a classical peroxidase catalytic cycle [29].

### Biotransformation of 4,2′,4′-trihydroxychalcone and 4,2′,4′-trihydroxy-3-methoxychalcone

The above results prompted us to examine whether the purified isoenzyme was still able to catalyse the biotransformation of chalcones **1a** and **1b** (Figure 1). 4,2′,4′-Trihydroxy-3-methoxychalcone (**1a**) was therefore treated with the acidic peroxidase in the presence of $H_2O_2$ as cofactor. The reaction was complete after 10 min as monitored by TLC and gave only one product (75 %), apparently coincident with the biflavanone (**3a** or **4a**) previously reported. Examination of NMR spectra revealed the crude product to be a mixture (3 : 1) of two compounds, which were separated by preparative TLC. The two products gave the same $M$H$^+$ (at 571 $m/z$) in the fast atom bombardment mass spectra and similar NMR spectra (Table 5). Notably, in the $^1$H NMR spectrum of the minor compound the signals for the protons H-2,H-2″ and H-3,H-3″ were missing and could be obtained only when the measurement was performed at 60 °C. This finding and the broadening of the resonance at 42 p.p.m. in the $^{13}$C NMR spectrum can be attributed to the restricted rotation about the C–C interflavonoid linkage.

The two compounds can thus be assigned the structure di(7,4′-dihydroxyflavanon-3-yl). Since H-2 and H-3 were in the *trans* relative position ($J = 12$ Hz) and $\alpha_D = 0$ for both the biflavanones one must be the racemate (**3a**) and the other the *meso* form (**4a**).

To determine the stereochemistry of the two biflavanones, a chiral NMR shift reagent, tris [3-(heptafluoropropylhydroxy-methylene)-(+)-camphorato]europium (III), was used in $^1$H

**Table 5** $^1$H and $^{13}$C NMR spectral data of 3,3″ biflavanones 3a and 4a obtained by the biotransformation of 4,2′,4′-trihydroxy-3-methoxychalcone

Spectra were measured in $C^2H_3COC^2H_3$, with tetramethylsilane as internal standard. In the $^1$H NMR spectra the signals showed the appropriate integrated intensities and multiplicities. Coupling constants (in Hz): $J_{2,3} = 12$, $J_{5,6} = 8.8$, $J_{6,8} = 2.5$, $J_{2',6'} = 2$, $J_{5',6'} = 8.2$.

| | 3a | | 4a | |
| --- | --- | --- | --- | --- |
| Position | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 2,2″ | 5.94 | 85.24 | 4.93 | 82.68 |
| 3,3″ | 2.74 | 52.01 | 3.72 | 49.97 |
| 4,4″ | – | 191.66 | – | 190.70 |
| 5,5″ | 7.70 | 129.78 | 7.77 | 129.91 |
| 6,6″ | 6.57 | 111.19* | 6.56 | 110.84 |
| 7,7″ | – | 165.32 | – | 165.40 |
| 8,8″ | 6.32 | 103.39 | 6.32 | 103.43 |
| 9,9″ | – | 164.08 | – | 164.22 |
| 10,10″ | – | 115.74 | – | 114.54 |
| 1′,1‴ | – | 129.99 | – | 129.71 |
| 2′,2‴ | 6.78 | 111.44* | 6.79 | 111.54 |
| 3′,3‴ | – | 148.47† | – | 148.22* |
| 4′,4‴ | – | 148.26† | – | 148.02* |
| 5′,5‴ | 6.83 | 115.21 | 6.86 | 115.19 |
| 6′,6‴ | 6.70 | 121.66 | 6.74 | 121.96 |
| 0 Me | 3.71 | 55.71 | 3.73 | 55.90 |

*, † Similarly marked signal assignments could be interchanged.

**Table 6** $^1$H and $^{13}$C NMR spectral data of 3,3″ biflavanones 3b and 4b obtained by the biotransformation of 4,2′,4′-trihydroxychalcone

Spectra were measured in $C^2H_3COC^2H_3$, with tetramethylsilane as internal standard. In the $^1$H NMR spectra the signals showed the appropriate integrated intensities and multiplicities. Coupling constants (in Hz): $J_{2,3} = 12$, $J_{5,6} = 8.5$, $J_{6,8} = 2.0$, $J_{2',3'} = J_{5',6'} = 8.5$.

| | 3b | | 4b | |
| --- | --- | --- | --- | --- |
| Position | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 2,2″ | 5.97 | 84.99 | 5.03 | 82.61 |
| 3,3″ | 2.73 | 51.81 | 3.73 | 49.93 |
| 4,4″ | – | 191.61 | – | 190.62 |
| 5,5″ | 7.71 | 129.85 | 7.77 | 130.06 |
| 6,6″ | 6.58 | 111.28 | 6.59 | 111.48 |
| 7,7″ | – | 165.33 | – | 165.31 |
| 8,8″ | 6.35 | 103.45 | 6.28 | 103.45 |
| 9,9″ | – | 164.22 | – | 164.26 |
| 10,10″ | – | 115.26 | – | 117.79 |
| 1′,1‴ | – | 129.20 | – | 129.28 |
| 2′,6′,2‴,6‴ | 7.01 | 130.13 | 7.12 | 130.67 |
| 3′,5′,3‴,5‴ | 6.86 | 116.24 | 6.88 | 116.24 |
| 4′,4‴ | – | 159.05 | – | 159.13 |

NMR studies of the methyl ethers **3c** and **4c**, prepared by treatment with $CH_2N_2$. In the $^1$H NMR spectrum (in $C^2HCl_3$) in the presence of a half molar ratio of the chiral shift reagent, the hexamethoxyl derivative of the major compound showed a splitting of the signals due to the methyne protons and was thus assigned the racemic structure **3c**.

Accordingly, the methyl ether of the minor compound gave one set of signals with no splitting, even in the presence of one molar ratio of the chiral shift reagent, and was assigned the *meso* form **4c**.

The biotransformation of 4,2′,4′-trihydroxychalcone (**1b**) by crude peroxidase from *C. didymobotrya* gave analogous results: together with the flavone **2b** [10], two biflavanones ($M$H$^+$ at 511 $m/z$, $^1$H and $^{13}$C NMR spectra in Table 6) in the ratio 3:1 were obtained. In comparison with the chemical shifts of H-2/H-2″ and H-3/H-3″ reported for di(5,7,4′-trihydroxyflavanone-3-yl) [30], i.e. 5.92 and 2.97 p.p.m. (D,L) and 4.92 and 3.94 p.p.m. (*meso*), the two compounds were assigned the stereostructures **3b** (major) and **4b** (minor).

4,2′,4′,Trihydroxychalcone (**1b**) has been reported to give, with HRP-C at different pH values (7.5–7.8), a series of oxidation products [31,32] but no dimeric compound. Therefore chalcone **1b** was treated with HRP under the same conditions used for *Cassia* peroxidase. As a result, the products **2b**, **3b** and **4b** (the last two in a slightly different ratio, 7:3) were again obtained in a comparable overall yield (45%).

## Conclusion

The purified peroxidase isoenzyme has allowed us to shed some light on the biogenetic pathway that leads to biflavanones from the corresponding chalcones. Notably, the results strongly suggest that chalcones are the direct precursors of the biflavanones [10], when either crude or purified peroxidases derived from cell cultures of *C. didymobotrya* are employed.

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