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# DIHYDROFLAVONOL 4-REDUCTASE ACTIVITY IN LIGNOCELLULOSIC TISSUES

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**Key Word Index**—*Picea abies*; *Pinus sylvestris*; *Larix decidua*; Pinaceae; dihydroflavonol 4-reductase; extraction; assay; bark; sapwood; conifers.

**Abstract**—Two enzyme assays using either radiolabelled or non-labelled substrates were applied to detect dihydroflavonol 4-reductase (DFR) activity in lignocellulosic tissues. The successful detection of DFR in these tissues rich in polyphenols relied on the addition of sufficient amounts of polyethylene glycol. DFR activity is measured in sapwood and inner bark of different conifer species. © 1997 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

Dihydroflavonol 4-reductase (DFR) catalyses the reduction of dihydroflavonols into leucoanthocyanidins (Fig. 1), one of the last steps in anthocyanin and proanthocyanidin biosynthetic pathways [1]. DFR has been identified in numerous tissues, including flower petals, barley grains and cell cultures, but not yet in lignocellulosic tissues such as bark or wood. Lignocellulosic tissues are often rich in polyphenols, making the extraction of enzymes from these tissues difficult and the development of complex extraction media necessary [2–4]. The methods of DFR extraction and assay have thus been optimized and applied to the detection of DFR in woods and barks of various conifers.

## RESULTS AND DISCUSSION

### *Dihydroflavonol* 4-*reductase assay*

DFR activity was assayed by detection of the leucoanthocyanidins produced by reduction of dihydroflavonol substrates (Fig. 1). Two different methods were used for their detection and separation: one is based on the use of [$^3$H]-(+)-dihydrokaempferol (DHK) as substrate and the detection of [$^3$H]-3,4-*cis*-leucopelargonidin (LPG) on TLC plates (Fig. 2); the other method uses (+)-dihydroquercetin (DHQ) substrate and detection of the 3,4-*cis*-leucocyanidin (LCY) product by reverse-phase HPLC.

The DFR assay with [$^3$H]-DHK could be used for detection of DFR activity but not for quantitative determination owing to a slow exchange of tritium with protons in buffers. The rate of exchange was found to depend on the nature of the buffer. About 25% of the DHK label was lost in Tris (pH 7) and phosphate (pH 7.3) buffers, and 50% in HEPES buffer (pH 7) within 2 hr. However, the method using the labelled substrate was useful for the detection of DFR activity due to its high sensitivity which was 20 times higher than that of the HPLC method.

### *Extraction of dihydroflavonol* 4-*reductase activity from lignocellulosic tissues*

The extraction of enzymes for plant tissues rich in tannins and other phenolic extractives (bark, many woods, etc.) is often critical for the successful detection of their activities. Polyphenol adsorbants, oxidase inhibitors and/or reducing agents are regularly added to the extraction buffer in order to limit the complexation of polyphenols with enzymes [5, 6]. The effect of adding ascorbic acid, calcium chloride and polyethylene glycol (PEG) on the extraction of protein and DFR from larch inner bark was studied in more detail. Addition of ascorbic acid (0–200 μM) had no significant influence on the extraction of proteins and on the DFR activity measured. The presence of dithioerythritol, another reducing agent, in the buffer is therefore sufficient. Addition of calcium chloride (25 μM) increased the concentration of proteins by

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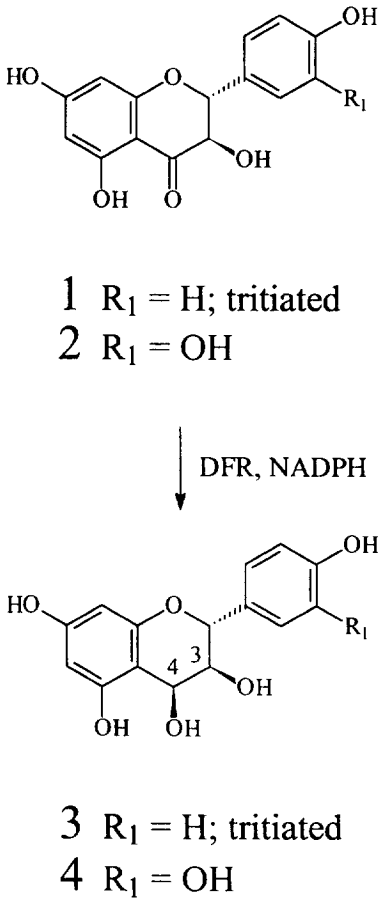


Fig. 1. Reduction of dihydroflavonols to leucoanthocyanidins by dihydroflavonol 4-reductase. **1**, [$^3$H]-dihydrokaempferol; **2** dihydroquercetin; **3**, [$^3$H]-3,4-*cis*-leucopelargonidin; **4**, 3,4-*cis*-leucocyanidin.

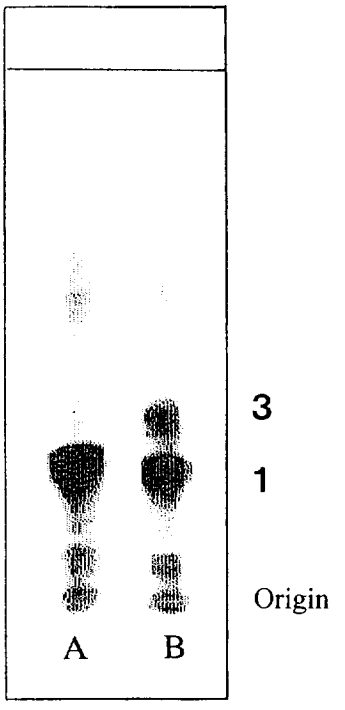


Fig. 2. Detection of dihydroflavonol 4-reductase activity by autoradiography. [$^3$H]-Dihydrokaempferol was treated with a protein extract from inner bark of European larch: lane A, no NADPH added; lane B, NADPH added. Products were separated on a cellulose thin layer plate with acetic acid–water (6:94) as eluent. For compounds **1** and **3**, see Fig. 1.

46% and that of DFR activity by 11%. Similar figures have been reported for chalcone synthase extraction from walnut bark [2]. Adding sufficient amounts of PEG (1.5%) was essential to increase the yields of protein extraction and to increase the levels of DFR activity detected (Fig. 3).

*Dihydroflavonol 4-reductase in wood and bark*

The extraction method and the DFR assay were then applied to lignocellulosic tissues. DFR was detected and estimated by the HPLC assay for DFR activity in the four inner bark samples examined (Table 1). However, the same method failed to detect DFR activity in outer, intermediate and inner sapwood of Douglas fir (*Pseudotsuga menziesii*) and European larch (*Larix decidua*), despite the known occurrence of procyanidins, although at low concentrations, which supports the existence of DFR in these tissues [7]. The DFR assay based on the use of radiolabelled DHK as substrate (detection limit 0.1 nkat $kg^{-1}$ bark) similarly failed to detect DFR activity in sapwood of these species.

## CONCLUSION

DFR activity is detected and estimated for the first time in lignocelluosic tissues. DFR activity measured in inner bark contributes to the biosynthesis of proanthocyanidins present in concentration as high as 90 mg $g^{-1}$ of bark [8]. Detection of DFR activity in sapwoods containing low amounts of proanthocyanidins (1 mg $g^{-1}$ in Douglas fir sapwood [7]) may require more sensitive methods than those developed here or a more careful examination of sapwood throughout the year. This may be due to an insufficient sensitivity of the assay or to seasonal variations in DFR activity. Variations along a season in the activity levels of several other enzymes in wood have been described [3, 4]. Other radiolabelled substrates, differing in the pattern of B-ring hydroxylation should also be tested as the structural requirements for substrates differ according to the source of the enzyme [9, 10].

## EXPERIMENTAL

*General.* Buffer A: HEPES 0.1 M, pH 7.3; PEG 1500 (Merck), 1.5%; sucrose, 10%; dithioerythritol, 1 mM; ascorbic acid, 100 mM; calcium chloride, 25 mM. Buffer B: potassium phosphate 0.1 M, pH 7.0; sucrose, 10%; sodium ascorbate, 20 mM. DHQ

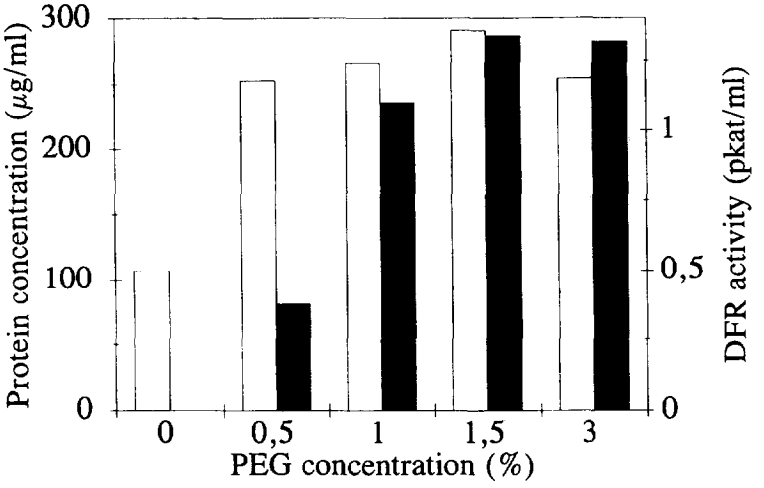


Fig. 3. Influence of the polyethylene glycol concentration in the extract medium on the protein content (white bars) and dihydroflavonol 4-reductase activity (black bars) in protein extracts from inner bark of European larch.

Table 1. Protein concentration and dihydroflavonol 4-reductase (DFR) activity in inner bark tissue of four conifers

| | Total extractable proteins (mg $g^{-1}$ bark) | DFR* (pkat $g^{-1}$ bark) |
|---|---|---|
| *Picea abies* (Norway spruce) | 1.28 | 7.3 |
| *Pinus sylvestris* (Scots pine) | 1.11 | 7.2 |
| *Larix decidua* (European larch) | 1.11 | 12.8 |
| *Pseudotsuga menziesii* (Douglas fir) | 1.05 | 2.2 |

* HPLC assay.

and DHK were purified from Douglas fir and European larch hardwoods, respectively [7]. [$^3$H]-DHK (5 Ci $mmol^{-1}$) was prepd by Amersham by catalytic exchange of aromatic protons with tritiated $H_2O$ of high specific activity (protocol TR.8) using unlabelled DHK isolated from heartwood of European larch. Proteins were determined by the Bradford method (Bio-Rad reagent) which shows limited interferences with polyphenols [11]. Bovine serum albumin was used as a standard.

*Leucoanthocyanidin preparation.* The 3,4-*trans* isomers of LPG and LCY were prepd by reduction of DHK and DHQ (10 mg), respectively, with sodium borohydride [12]. The crude products were extracted with EtOAc and divided into 10 aliquots. Each aliquot was added to a potassium phosphate buffer (50 mM, pH 6.8, 50 μl) and the organic solvent removed under red. pres. MeOH (950 μl) was added and the tubes stored at −80°. 3,4-*Trans*-leucoanthocyanidins (5 μl) were partially isomerised in $H_2O$ (95 μl) at 37° for 40 min to provide the standards required for the identification of the *cis*-isomers in the products of enzymic reduction. Under these conditions, 3,4-*trans*-LCY gave the 3,4-*cis*-isomer (10%) and undefined polymers (23%) (yields determined by HPLC at 280 nm after addition of 33 μl MeOH). LPG and LCY isomers were identified by comparison of their $R_f$ values (cellulose TLC) [9, 13] and retention times (HPLC) [14] with those previously published.

*Dihydroflavonol 4-reductase extraction.* Inner barks and sapwoods were collected in Oberschleissheim and Freising (Germany) on trunks of 12–60-year old trees, 1–2 m above ground, over a period extending from June to November. Samples were immediately plunged in liquid $N_2$, freeze-dried and stored under vacuum at 4°. They were red. to powder with a wood file when needed. The following extraction of DFR was carried out at 4°. Wood powder (200 mg) was further ground for 5 min in a chilled mortar with PVPP (Sigma, 200 mg), quartz sand (Merck, 200 mg) and buffer A (3 ml). After centrifugation (10 min at 20000 *g*), the supernatant was stirred for 15 min with Dowex 1X2-200 (Sigma) pre-equilibrated in the same buffer A. The resin was removed by centrifugation and the supernatant (0.5 ml) desalted over a Sephadex G-25 column (NAP-10, Pharmacia) pre-conditioned in buffer B. The crude enzyme extract was obtained by elution with 1 ml buffer B.

*Dihydroflavonol 4-reductase assays.* The crude enzyme extract (70 μl) was either mixed with DHQ (10 nmol in 5 μl MeOH) or with [$^3$H]-DHK (5 μCi in 5 μl EtOH), NADPH (Serva, 5 μmol in 10 μl $H_2O$), 6-phosphoglucose (Serva, 6 μmol in 10 μl $H_2O$), 6-phosphoglucose dehydrogenase (Serva, one unit in 5

$\mu$l $H_2O$) and kept at 30° during 60 min. Both the substrate concn and the reaction duration have been optimized. Controls were systematically made by omitting the addition of NADPH or denaturing the enzyme by heating the crude extract at 100° for 10 min. The products were then extracted by EtOAc ($2 \times 250$ $\mu$l). The soln was concd under red. pres. before TLC analysis. The radiolabelled products were sepd by cellulose TLC with *n*-BuOH satd with $H_2O$ or HOAc–$H_2O$ (6:94) as solvents. The compounds were detected with a Berthold TLC linear analyser (radiolabelled products) or by spraying dimethylaminocinnamaldehyde and sulphuric acid [15]. For HPLC analysis of the non-labelled products, K-Pi buffer (50 mM, pH 6.8, 90 $\mu$l) was added to the EtOAc extract, the organic solvent removed under red. pres. and MeOH (30 $\mu$l) added to the residual aq. phase. Leucoanthocyanidins were analysed in 20 $\mu$l aliquots on a Spherisorb ODS 11 RP18 column (Bischoff, $250 \times 4.6$ mm, 5 $\mu$m); eluent A, formic acid 1% and ammonium formate 0.05%; eluent B, MeOH; gradient 25–60% B in 18 min; flow, 1 ml $min^{-1}$; detection, 280 nm. Retention times were as follows: 3,4-*cis*-LCY, 4.2 min; 3,4-*trans*-LCY, 5.6 min; DHQ, 12.0 min. 3,4-*Cis*-LCY isomer concn was established by comparison with a 3,4-*trans*-LCY reference soln (both isomers have the same molar *A* at 278 nm [14]). Concn of the reference soln was determined by UV spectroscopy.

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