Lignans are a widely distributed class of natural products, whose functions and distribution suggest that they are one of the earliest forms of defense to have evolved in vascular plants; some, such as podophyllotoxin and enterodiol, have important roles in cancer chemotherapy and prevention, respectively.

Entry into lignan enzymology has been gained by the ~3000-fold purification of two isoforms of (+)-pinoresinol/(+)-lariciresinol reductase, a pivotal branchpoint enzyme in lignan biosynthesis. Both have comparable (−34.9 kDa) molecular mass and kinetic (V_max/K_m) properties and catalyze sequential, NADPH-dependent, stereospecific, hydride transfers where the incoming hydride takes up the pro-R position.

The gene encoding (+)-pinoresinol/(+)-lariciresinol reductase has been cloned and the recombinant protein heterologously expressed as a functional β-galactosidase fusion protein. Its amino acid sequence reveals a strong homology to isoflavone reductase, a key branchpoint enzyme in isoflavonoid metabolism and primarily found in the Fabaceae (angiosperms). This is of great evolutionary significance since both lignans and isoflavonoids have comparable plant defense properties, as well as similar roles as phytoestrogens. Given that lignans have comparable plant defense properties, as well as similar roles as phytoestrogens, as its etoposide and teniposide derivatives, is one of the very few examples of plant anticancer agents successfully employed (1). Antiviral properties have also been reported, e.g. (-)-arctigenin (16), (-)-trachelogenin (16), and nordihydroguaiaretic acid (17) are effective against HIV due to their pronounced reverse transcriptase inhibitory activities. Some lignans, e.g. matairesinol (18), inhibit cAMP-phosphodiesterase, whereas others enhance cardiovascular activity, e.g. syringaresinol β-d-glucoside (19). There is also a high correlation between the presence of the “mammalian” lignans or “phytoestrogens,” enterolactone and enterodiol, formed following digestion of high fiber diets, and the reduced incidence rates of breast and prostate cancers (so-called chemoprevention) (20).

The biosynthetic pathways to the lignans are only now being defined, although no purification of any enzyme, or the cloning of any gene, in their pathways has hitherto been reported. Based on radiolabeling experiments with crude enzyme extracts from Forsythia intermedia, it was established that entry into the 8,8'-linked lignans, which represent the most prevalent dilligol linkage known (21), occurs via stereoselective coupling of two achiral coniferyl alcohol molecules to afford (+)-pinoresinol (22, 23), a furufuran lignan. In F. intermedia, and presumably other species, (+)-pinoresinol undergoes sequential reduction to generate (+)-lariciresinol and then (+)-seco-isolariciresinol (24, 25). (-)-Matairesinol is subsequently formed via dehydration of (+)-secoisolariciresinol, further metabolism of which presumably affords lignans such as the antiviral (-)-trachelogenin in Ipomoea carthaca and (-)-podophyllotoxin in Podophyllum peltatum (Fig. 1). Thus, the reductive steps giving (+)-lariciresinol and (+)-secoisolariciresinol are pivotal points in lignan metabolism, since they represent entry into the furano, dibenzyllbutane, dibenzyllbutyrolactone, and aryltetrahydronaphthalene lignan subclasses. This paper describes the purification and characterization of (+)-pinoresinol/(+)-lariciresinol reductase from F. intermedia, the cloning of its cDNA, and the heterologous expression of its recombinant protein.

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β-galactosidase fusion protein in catalytically active form in *Escherichia coli*.

**EXPERIMENTAL PROCEDURES**

**Plant Materials—** *F. intermedia* plants were either obtained from Bailey’s Nursery (var. Lynnwood Gold, St. Paul, MN), and maintained in Washington State University greenhouse facilities, or were gifts from the local community.

**Materials—** All solvents and chemicals used were reagent or HPLC grade. Unlabeled (-)-pinosinol and (-)-larciresinol were synthesized as described (24). [4R]-HINADPH was obtained as previously reported (25) by modification of the procedure of Moram et al. (26), and [4R]-HINADPH was prepared according to Anderson and Lin (27). Yeast glucose-6-phosphate dehydrogenase (type IX, 22.32 mmol h⁻¹ mg⁻¹) and yeast hexokinase (type F300, 15.12 mmol h⁻¹ mg⁻¹) were purchased from Sigma, and dihydrofolate reductase (Lactobacillus casei, 33.48 mmol h⁻¹ mg⁻¹) was obtained from Biopure Co. Affi-Gel Blue Gel (100–200 mesh) and Bio-Gel HT hydroxyapatite were purchased from Bio-Rad, and phenyl-Sepharose CL-4B, Mono Q HR 5/5, Mono P HR 5/20, Superose 6, Superose 12, Superdex 75, PD-10 columns, molecular weight standards, and Polybuffer 74 were obtained from Pharmacia. LKB Biotechnology, Inc. Adenosine 2′,3′-diphosphate-Sepharose and Reactive Yellow 3-Agarose were from Sigma. Taq thermostable DNA polymerase was obtained from Perkin-Elmer Cetus, and restriction enzymes were from Life Technologies, Inc. (HaelIII), Boehringer Mannheim (Sau3A), and Promega (TaqI). pT7Blue T-vector and competent NovaBlue cells were purchased from Novagen, and radiolabeled nucleotides (labeled 32P)ATP and [γ-32P]-ATP were from DuPont NEN.

Oligonucleotide primers for polymerase chain reaction (PCR) and sequencing were synthesized by Life Technologies, Inc. GeneClean II™ kits (Bio-101 Inc.) were used for purification of PCR fragments, with the gel-purified DNA concentrations determined by comparison to a low DNA mass ladder (Life Technologies, Inc.) in 1% agarose gels.

**Instrumentation—** H nuclear magnetic resonance spectra (300 and 500 MHz) were recorded on Bruker AMX300 and Varian VXR500/80 spectrometers, respectively, using CDCl₃ as solvent with chemical shifts (δ ppm) reported downfield from tetramethylsilane (internal standard). UV (including RNA and DNA determinations at A₂₆₀) and mass spectra were obtained on Lambda 6 UV/VIs and VG 7070E (ionizing voltage 70 eV) spectrophotometers, respectively. High performance liquid chromatography was carried out using either reversed-phase (Waters, Nova-pak C₁₈, 150 × 3.9-mm inner diameter) or chiral (Daicel, Chiralcel OD or Chiralcel OC, 250 × 4.6-mm inner diameter) columns, with detection at 280 nm (25). Radioactive samples were analyzed in Ecolume (ICN) and measured using a liquid scintillation counter (Packard, Tricarb 2000 CA). A Temptron II thermocycler (Thermolyne) was used for all PCR amplifications. Purification of DNA for sequencing employed a QIAwell Plus plasmid purification system (Thermolyne) was used for all PCR amplifications. Purification of DNA was performed using the original protocol provided by the manufacturer. A Promega genomic DNA mass ladder (Life Technologies, Inc.) in 1.5% agarose gels.

**Spectrometers, respectively, using CDCl₃ as solvent with chemical**

**Hydroxyapatite II Chromatography—** Active fractions (160 mg, 91 nmol larciresinol) from the preceding step were applied to a hydroxyapatite column (3 cm × 1.5 cm), equilibrated in 10 mM potassium phosphate buffer, pH 7.8, containing 5 mM dithiothreitol (buffer B). Larciresinol was eluted with a linear gradient of decreasing concentration of NaCl (5–0 M) in buffer B at a flow rate of 1 ml min⁻¹. Active fractions were subjected to reversed-phase chromatography (Waters, Nova-pak C₁₈, 150 × 3.9-mm inner diameter, equilibrated in 10 mM potassium phosphate buffer, pH 7.8, containing 5 mM dithiothreitol). Pinoresinol/lariciresinol reductase activity (0.85 mg, 1,051 nmol h⁻¹) was next loaded on a 2′,3′-diphosphate-Sepharose column (1 × 10 cm) previously equilibrated in buffer A containing 5 mM NaCl. The column was washed with two bed volumes of the same buffer. Pinoresinol/lariciresinol reductase was eluted using a linear gradient of decreasing concentration of NaCl (5–0 M) in 40 ml in buffer A at a flow rate of 1 ml min⁻¹. Fractions catalyzing pinoresinol/lariciresinol reductase activity were pooled and concentrated to 1 ml using a Centriplus 10 microconcentrator (Amicon, Beverly, MA).

**Hydroxyapatite I Chromatography—** Active protein (31 mg, 91 nmol larciresinol) from the preceding step was applied to a hydroxyapatite column (3 cm × 1.5 cm), equilibrated in 10 mM potassium phosphate buffer, pH 7.0, containing 5 mM dithiothreitol (buffer B). Larciresinol was eluted with a linear gradient of decreasing concentration of NaCl (5–0 M) in 40 ml in buffer A at a flow rate of 1 ml min⁻¹. Active fractions were pooled and concentrated to 1 ml using a Centriplus 10 microconcentrator (Amicon, Beverly, MA).

**Hydroxyapatite II Chromatography—** The resulting enzyme solution (6.5 mg, 463 nmol h⁻¹) was next loaded on a 2′,3′-diphosphate-Sepharose column (1 × 10 cm), previously equilibrated in buffer A containing 2.5 mM EDTA (buffer A¹), and then washed with 25 ml of buffer A. Pinoresinol/lariciresinol reductase was eluted with a step gradient of NaCl (0.3 M in 10 ml in buffer A) at a flow rate of 0.5 ml min⁻¹. NAD⁺ (up to 3 mM) did not elute pinoresinol/lariciresinol reductase activity. Because of the interference of the absorbance of the NAD⁺, it was not possible to directly monitor the eluent at 280 nm. Protein concentrations for each fraction were determined spectrophotometrically according to Bradford (29).

**Hydroxyapatite III Chromatography—** Fractions exhibiting pinoresinol/lariciresinol reductase activity (0.85 mg, 1,051 nmol h⁻¹) were combined and directly applied to a second hydroxyapatite column (1 × 3 cm), equilibrated in buffer B, with the enzyme eluted with a linear gradient of potassium phosphate buffer, pH 7.0 (0.01–0.4 M in 45 ml), at a flow rate of 1 ml min⁻¹. Active fractions were pooled and concentrated to 1 ml using a Centriplus 10 microconcentrator (Amicon, Beverly, MA).

**Fast Protein Liquid Chromatography (Superose 12 and Mono Q Chromatography)—** Combined fractions from the preceding step having the highest activity (50 µg, 10,940 nmol h⁻¹) were pooled and concentrated to 1 ml, using a Centricon 10 microconcentrator (Amicon, Beverly, MA).

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¹ The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); Bis-Trips, 2-[bis(2-hydroxyethyl)amino]-2′-hydroxymethyl-propane-1,3-diol; pfu, plaque-forming units; ORP, open reading frame; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
Inc.). The enzyme solution was then applied in portions of 200 μl to a fast protein liquid chromatography (FPLC) Biogel P-10 column. Gel filtration was performed in a buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5 mM dithiothreitol at a flow rate of 0.4 ml min⁻¹. Pinoresinol/lariciresinol reductase was eluted with 12.8 ml of the mobile phase. The active fractions that coincided with the UV profile (absorbance at 280 nm) were pooled (20 μg, 15,300 nmol h⁻¹ mg⁻¹) and desalted (PD-10 prepacked columns). This was next applied to a Mono Q HR 5/5 column (Pharmacia), equilibrated in buffer A. The column was washed with 10 ml of buffer A and pinoresinol/lariciresinol reductase activity eluted using a linear NaCl gradient (0–500 mM in 50 ml) in buffer A at a flow rate of 0.5 ml min⁻¹. Aliquots (30 μl) of the collected fractions were analyzed by SDS-polyacrylamide gel electrophoresis, using a gradient (4–15% acrylamide) gel. Proteins were visualized by silver staining. The active fractions (34–57, 27,750 nmol h⁻¹ mg⁻¹) and 38–41 (30,790 nmol h⁻¹ mg⁻¹) were pooled separately and immediately used for characterization.

**pH and Temperature Optima**—To determine the pH optimum of pinoresinol/lariciresinol reductase, standard assay conditions were employed except that the buffer was replaced with 50 mM Bis-Tris propane buffer in the pH range of 6.3–9.4. The temperature optimum was examined in the range between 4 and 50 °C under standard assay conditions.

**pl Determination**—The isoelectric point of pinoresinol/lariciresinol reductase was estimated by chromatofocusing on a Mono P HR 5/20 FPLC column. For this purpose, active fractions from the Superose 12 gel filtration column were pooled and the exchanged with 25 mM Bis-Tris protonated HCl, pH 7.0, equilibrated in the same buffer. The preparation so obtained was loaded on the chromatofocusing column, and a pH gradient between 7.1 and 3.9 was formed, using Polybuffer 74 as eluent at a flow rate of 0.5 ml min⁻¹. Fractions (40 μl) of the collected fractions were analyzed by SDS-polyacrylamide gel electrophoresis, using a gradient (4–15% acrylamide) gel. Proteins were visualized by silver staining. The active fractions (40–41) containing 30,790 nmol h⁻¹ mg⁻¹ were pooled separately and immediately used for characterization.

**Kinetic Parameters**—Initial velocity studies were performed in triplicate experiments, using 50 mM Bis-Tris propane buffer, pH 7.4, containing 5 mM dithiothreitol, pure enzyme (after MonoQ anion-exchange chromatography), and 10 different substrate concentrations (between 8.5 and 80 mM) in 22 ml of the same buffer and incubated at 37 °C for 1 h. Kinetic parameters were determined from Lineweaver-Burk plots.

**Enzymatic Formation of (+)-lariciresinol**—An aqueous solution of (+)-pinoresinol (5.2 mM in MeOH, 4 ml) was added to Tris-HCl buffer (20 mM, pH 8.0, containing 5 mM dithiothreitol, 22 ml) and (l4R)-3HINADHPH (20 μM in H2O, 4 ml), with the whole added to the enzyme preparation (20 ml). After incubation at 30 °C for 1 h with shaking, the assay mixture was extracted with 25 mM Bis-Tris protonated HCl, pH 7.0, and solubles were combined, washed with saturated NaCl (50 ml), dried (Na2SO4), and evaporated to dryness in vacuo. The resulting extract was reconstituted in a minimum amount of ETOAc, applied to a silica gel column (0.5 × 7 cm), and eluted with ETOAc/hexanes (1:2). Fractions containing the enzymatic product were combined and evaporated to dryness to give (+)-l[(-)3H]lariciresinol (1.8 mg, 18%) as an amorphous powder. 1H NMR (300 MHz) (CDCl3): 2.39 (m, 1H, CH2), 2.71 (m, 1H, C6-H), 2.88 (d, 1H, JF2,b = 5.0 Hz, C4-H), 3.73 (dd, 1H, JF1,a = 7.0 Hz, JF2,b = 8.5 Hz, C6-H), 3.76 (dd, 1H, JF1,a = 6.5 Hz, JF2,b = 8.5 Hz, C6-H), 3.86 (s, 3H, OCH3), 3.88 (s, 3H, OCH3), 3.92 (dd, 1H, JF1,a = 6.6 Hz, C5-H), 6.68–6.70 (m, 2H, ArH), 6.75–6.85 (m, 4H, ArH); MS m/z (%): 361 (M⁺-H) 51 (7), 294 (75), 279 (12), 233 (22), 215 (30), 185 (100), 173 (17). The resulting mixture was subjected to reversed-phase HPLC analysis (C-8 column, Applied Biosytems), this being eluted with a linear gradient over 2 h from 0 to 100% acetonitrile (in 0.1% TFA) at a flow rate of 0.2 ml/min with detection at 214 nm. Fractions containing individual oligopeptide peaks were collected manually and directly submitted to amino acid sequencing. Cyanogen bromide digestion was performed by incubation of the reductase (150 pmol) with 0.5 mM cyanogen bromide in 70% acetic acid for 4 h at room temperature, following which the reaction was exhaust. The resulting oligopeptide fragments were separated by HPLC and sequenced (see "Results and Discussion").

**Library Screening**—600,000 pfu of *F. intermedia* amplified cDNA library were plated for primary screening, according to Stratagene's instructions. Insert sizes were determined using the rapid boiling lysis and PCR technique (with R20-mer and U19-mer primers) according to the manufacturer's instructions. Restriction analysis was performed to determine whether all inserts for each primer pair (PLRN5 + PLRI5R or PLRN5 + PLRI4R) were the same, as follows: to 20 μl each of a 100-μl PCR reaction (insert of interest amplified with R20-mer and U19-mer primers) were added 4 units of HaeIII, 1.5 units of Sau3A, or 5 units of TaqI restriction enzyme. Restriction digestions were allowed to proceed for 1 h at 37 °C and 37 °C for TaqI reactions. Restriction products were resolved in 1.5% agarose gels giving one restriction group for all inserts tested. Three recombinant plasmids from PLRN5 + PLRI5R (called pT7PLR1 – pT7PLR3) and two recombinant plasmids from PLRN5 + PLRI4R (called pT7PLR4 and pT7PLR5) PCR products were selected for DNA sequencing; all contained the same opening frame (ORF). The (+)-pinoresinol/(-)-lariciresinol reductase probe was next constructed as follows: five 100-μl PCR reactions were performed as above with PLRN5 + PLRI5R and PLRN5 + PLRI4R primer pairs. The five reactions from each primer pair were microconcentrated (Microcon 30, Amicon Inc.) and washed with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 × 200 μl), with the PCR products subsequently recovered in TE buffer (2 × 50 μl). These were resolved in preparative 1.5% agarose gels. Each gel-purified PCR product (~0.2 pmol) was then ligated into the pT7Blue T-vector and transformed into competent NovaBlue cells, according to Novagen's instructions. Insert sizes were determined using the rapid boiling lysis and PCR technique (with R20-mer and U19-mer primers) according to the manufacturer's instructions. Restriction analysis was performed to determine whether all inserts for each primer pair (PLRN5 + PLRI5R or PLRN5 + PLRI4R) were the same, as follows: to 20 μl each of a 100-μl PCR reaction (insert of interest amplified with R20-mer and U19-mer primers) were added 4 units of HaeIII, 1.5 units of Sau3A, or 5 units of TaqI restriction enzyme. Restriction digestions were allowed to proceed for 1 h at 37 °C and 37 °C for TaqI reactions. Restriction products were resolved in 1.5% agarose gels giving one restriction group for all inserts tested. Three recombinant plasmids from PLRN5 + PLRI5R (called pT7PLR1 – pT7PLR3) and two recombinant plasmids from PLRN5 + PLRI4R (called pT7PLR4 and pT7PLR5) PCR products were selected for DNA sequencing; all contained the same opening frame (ORF). The (+)-pinoresinol/(-)-lariciresinol reductase probe was next constructed as follows: five 100-μl PCR reactions were performed as above with 10 ng of pT7PLR3 DNA (containing the PLRN5-PLRI5R insert) with primers PLRN5 and PLRI5R. Gel-purified pT7PLR3 insert (50 ng) was used with Pharmacia's QuickPrime kit and [α-32P]dCTP, according to kit instructions, to perform a radiolabeled probe (0.1 ml), which was purified over BioSpin 6 columns (Bio-Rad) and added to carrier DNA (0.5 mg/ml sheared salmon sperm DNA (Sigma), 0.9 ml).

**Library Screening**—600,000 pfu of *F. intermedia* amplified cDNA library were plated for primary screening, according to Stratagene's instructions. Plaques were blotted onto Volley Nylon membrane circles (Micron Separations Inc.), which were then allowed to air dry. The membranes were placed between two layers of Whatman's 3MM Cellulose Nitrate paper. cDNA Library Phase DNA was fixed to the membranes and denatured in one step by autoclaving for 2 min at 100 °C with fast exhaust. The membranes were washed for 30 min at 37 °C in 6 × standard saline citrate (SSC) and 0.1% Sds and prehybridized for 5 h with gentle shaking at 57–58 °C in preheated 6 × SSC, 0.5% Sds, and 5 × Denhardt's reagent (hybridization solution, 32P in a 15 ml hybridization dish (190 × 75 mm). The 32P-radiolabeled probe (see above) was denatured (boiling, 10 min), quickly cooled (ice, 15 min), and added to a preheated fresh hybridization solution (60 ml, 58 °C) in a crystallization dish (150 × 75 mm). The prehybridized membranes were next added to this dish, which was then covered with plastic wrap. Hybridization was performed for 18 h at 55–58 °C with gentle shaking. The membranes
The DNA was transferred to charged nylon membranes (GeneScreen Plus®, DuPont NEN), cross-linked to the membrane (Stratalinker from Stratagene), prehybridized, hybridized with a 32P-degenerate oligonucleotide (primer PLRI4F) as the probe and washed according to the membrane manufacturer's recommendations for aqueous hybridization conditions. The membrane was then exposed to Kodak X-OMAT AR film for 48 h at −80 °C with intensifying screens. For RNA gel blot analysis, total RNA (30 μg per lane) from F. intermedia stem tips was separated by size by non-denaturing agarose gel electrophoresis. The RNA was transferred to charged nylon membranes (GeneScreen Plus®, DuPont NEN) and visualized as above for the DNA gel blot analysis. The probe for RNA gel blot analysis was the same as that used for library screening (see above).

Expression in E. coli—Purified plasmid DNA from pPLR3 (ORF in frame with the β-galactosidase gene α-complementation particle in pBluescript) was transformed into NovaBlue cells according to NovaGen's instructions. Transformed cells (5-ml cultures) were grown at 37 °C with shaking (225 rpm) to mid log phase (A600 = 0.5) in LB medium (28) supplemented with 12.5 μg ml−1 tetracycline and 50 μg ml−1 ampicillin. IPTG (isopropyl β-D-thiogalactopyranoside) was then added to a final concentration of 10 mM, and the cells were allowed to grow for 2 h. Cells were collected by centrifugation and resuspended in 500 μl (per 5-ml culture tube) of buffer (20 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol). Lysozyme (5 μl of 0.1 mg ml−1, Research Organics, Inc.) was next added, and following incubation for 10 min, the cells were

![Image of proposed biosynthetic pathway from E-coniferyl alcohol to selected lignans representing main structural types of the 8,8'-linked lignans.](image-url)

**Fig. 1.** Proposed biosynthetic pathway from E-coniferyl alcohol to selected lignans representing main structural types of the 8,8'-linked lignans.

**Table I.** Purification table for (+)-pinoresinol/(-)-lariciresinol reductase from F. intermedia

| Purification step                  | Protein | Total activity | Specific activity | Recovery | Purification factor |
|-----------------------------------|---------|----------------|------------------|----------|--------------------|
| 40-60% (NH4)2SO4 fractionation    | 1913    | 8,737          | 5                | 100      | 1                  |
| Affi-Blue                         | 150     | 7,607          | 51               | 87       | 10                 |
| Phenyl-Sepharose                  | 31      | 2,786          | 91               | 32       | 18                 |
| Hydroxyapatite I                  | 6.5     | 3,006          | 463              | 34       | 93                 |
| ADP-Sepharose                     | 0.85    | 893            | 1,051            | 10       | 210                |
| Hydroxyapatite II                 | 0.16    | 1,274          | 7,960            | 15       | 1,553              |
| Affi-Yellow                       | 0.05    | 547            | 10,940           | 6        | 2,188              |
| Superose 12                       | 0.02    | 306            | 15,300           | 3.5      | 3,060              |
lysed by sonication (3 × 15 s). After centrifugation at 14,000 × g at 4 °C for 10 min, the supernatant was removed and assayed for (+)-pinoresinol/(+)-lariciresinol reductase activity (210 μl supernatant per assay) as described above. Controls included assays of pPLR2 (cDNA out of frame) with all assay components, as well as pPLR3 and pPLR2 with no substrate except [4(2R)-3H]NADPH. Separation of products and chiral identification were performed by HPLC as described previously (25).

RESULTS AND DISCUSSION

Purification and Characterization of (+)-Pinoresinol/(+)-Lariciresinol Reductase—From our previous investigations (24) using crude enzyme extracts from F. intermedia, it was established that soluble, NADPH-dependent, reductases were capable of converting the sequential conversion of (+)-pinoresinol into (+)-lariciresinol and (−)-secoisolariciresinol, respectively (Fig. 1). While it was unclear whether more than one reductase was required to catalyze the sequential steps, the reductions proceeded via abstraction of the pro-R hydride of NADPH, resulting in an “inversion” of configuration at both the C-7 and C-7′ positions of the products, (+)-lariciresinol and (−)-secoisolariciresinol (25).

In the further study of these lignan branch point enzymes, the first objectives were the purification and characterization of the protein(s) involved. As shown in Table I, purification was achieved via an eight-step procedure involving three types of affinity chromatography, as well as hydrophobic interaction, hydroxyapatite, and gel filtration steps. This resulted in a 3060-fold purification of (+)-pinoresinol/(+)-lariciresinol reductase. As for many of the enzymes involved in phenylpropanoid metabolism, the protein was in very low abundance, i.e. 20 kg of F. intermedia stems yielded only ~20 μg of the purified (+)-pinoresinol/(+)-lariciresinol reductase. Various permutations of this overall protocol were also examined, but none produced better results than that given. Interestingly, in all stages of the purification protocol, (+)-pinoresinol/(+)-lariciresinol reductase activities co-eluted.

Given this observation, it was next essential to unambiguously ascertain whether more than one form of the protein existed. To this end, the 3060-fold purified protein was subjected to chromatofocusing on a Mono P column, using Polybuffer 74 as eluent, where again only a single peak of activity (corresponding to pI 5.7) was noted. Subsequent application of this preparation to a SDS-gradient gel electrophoresis (4–15% polyacrylamide) revealed the presence of two protein bands of similar apparent molecular weight (Fig. 2A, lane 2), whose separation was achieved via anion-exchange chromatography on an Mono Q HR 5/5 matrix (Fig. 2B). As can be seen from SDS-PAGE analysis (Fig. 2A, lanes 3–9), they had apparent molecular masses of ~36 and ~35 kDa, respectively.

Molecular Weight—Native molecular weights of each reductase were estimated via comparison of their elution behavior to calibrated molecular weight standards obtained by analysis of their elution profiles on Superose 12, Superose 6, and Superdex 75 gel filtration FPLC columns (see “Experimental Procedures”). For each reductase, an apparent native molecular weight of 59,000 was calculated based on its elution volume, in contrast to that of ~36,000 and ~35,000 by SDS-polyacryl-

![Fig. 2. Separation of two isofunctional forms of (+)-pinoresinol/(+)-lariciresinol reductase on Mono Q anion-exchange column. Panel A, silver-stained SDS-PAGE (4–15% gel) of active fractions: lane 1, molecular mass standards; lane 2, load; lanes 3–9, fractions 35–41. Molecular mass standards (Pharmacia Biotech, Inc.): phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). Panel B, elution profile of enzymatic activities](image)

![Fig. 3. Stereospecificity of hydride transfer during reduction of (+)-pinoresinol and (+)-lariciresinol.](image)
amide gel electrophoresis (Fig. 2A). While the discrepancy between molecular weights from gel filtration and SDS-PAGE remains unknown, it can tentatively be proposed that although the native protein likely exists as a dimer, it could also be a monomer of asymmetric shape, thereby altering its effective Stokes radius (36–39), as reported for human thioredoxin reductase and yeast metalloendopeptidase.

**pf Determinations and pH/ Temperature Optima**—The pf values were determined by chromatofocusing on a Mono P column, using Polybuffer 74 as eluent, over the pH range of 3.9–7.1. Each reductase form had a pf 5–7. The pH optimum was examined over the range of pH 6.3 to 9.4 and found to be pH 7.4. At optimum pH, the temperature optimum for the reductase activity was established to be −30 °C.

**Kinetic Properties**—It was instructive to ascertain whether the two reductase forms catalyzed distinct reductions, i.e. that of the conversion of (+)-pinoresinol to (+)-lariciresinol or (+)-lariciresinol to (+)-secoisolariciresinol, respectively, or whether either displayed a preference for (+)-pinoresinol or (+)-lariciresinol as substrates. To this end, initial velocity studies were carried out, individually employing both (+)-pinoresinol and (+)-lariciresinol as substrates in concentrations ranging between 8.8 and 160 μM, while keeping the NADPH concentration constant at 80 μM. Apparent kinetic constants were obtained from Lineweaver-Burk plots. Importantly, they were essentially the same for both forms (i.e. K<sub>m</sub> for pinoresinol, 27 ± 1.5 and 23 ± 1.3 μM; K<sub>m</sub> for lariciresinol, 121 ± 5.0 and 123 ± 6.0 μM). In an analogous manner, apparent maximum velocities (expressed as μmol h<sup>−1</sup> mg<sup>−1</sup> of protein) were also essentially identical (i.e. V<sub>max</sub> for pinoresinol, 16.2 ± 0.4 and 17.3 ± 0.5; for lariciresinol, 25.2 ± 0.7 and 29.9 ± 0.7). Thus, all available evidence suggests that (+)-pinoresinol/(+)-lariciresinol reductase exists as two isoforms, with each catalyzing the reduction of both substrates. How this reduction is carried out, i.e. whether both reductions are done in tandem, in either quinone or furano ring form will await further study using a more abundant protein source.

**Stereo specificity of Hydride Transfer**—Since the two (+)-pinoresinol/(+)-lariciresinol reductase isoforms exhibited essentially identical catalytic characteristics, a preparation containing both isoforms was used to examine the stereospecificity of the hydride transfer. The strategy adopted utilized selective deuterium labeling using NADPH as cofactor for the reduction of (+)-pinoresinol, with the enzymatic product, (+)-lariciresinol, being analyzed by H NMR and mass spectroscopy. Thus, (+)-pinoresinol were incubated with the enzyme preparation in the presence of stereospecifically deuto-labeled [4R-(2H)]NADPH prepared via the method of Anderson and Lin (27). The enzymatic product was established to be (+)-(7'R)-2H]lariciresinol, as evidenced by the disappearance of the 7'-pro-R proton at δ 2.51 ppm due to its replacement by deuterium and by its molecular ion at (m/z) 361 (M'<sup>+</sup> + 1) corresponding to the presence of one deuterium atom at C-7' (see “Experimental Procedures”). Thus, hydride transfer from (+)-pinoresinol to (+)-lariciresinol had occurred in a manner whereby only the 7'-pro-R hydrogen position of (+)-lariciresinol was deuterated. An analogous situation was observed for the conversion of (+)-lariciresinol into (+)-secoisolariciresinol, thereby establishing that the overall hydride transfer was completely stereospecific (Fig. 3).

**Cloning of the cDNA Encoding (+)-Pinoresinol/(+)-Lariciresinol Reductase**—With the purified (+)-pinoresinol/(+)-lariciresinol reductase on hand, our next objectives were to prepare the corresponding Forsythia cDNA library and to obtain the gene(s) encoding the enzyme of interest. But, initial attempts to isolate functional F. intermedia RNA from fast-growing green stem tissue were unsuccessful, due to difficulties encountered via facile oxidation by its plant phenolic constituents. This problem was, however, successfully overcome by adaptation of an RNA isolation procedure, specifically designed for woody plant tissue, which uses low pH and reducing conditions in the extraction buffer to prevent oxidation (32). Poly(A)<sup>+</sup> mRNA (5 μg) thus obtained was used to construct a λZAP F. intermedia stem cDNA library (total titer of 1.2 × 10<sup>6</sup> pfu), which was subsequently amplified (158 ml, 1.2 × 10<sup>10</sup> pfu/ml). From an aliquot (30 ml) of the amplified library, the purified F. intermedia cDNA library DNA needed for subsequent PCR experimentation was isolated.

Degenerate primers (underlined in Fig. 4) for PCR amplification of the F. intermedia (+)-pinoresinol/(+)-lariciresinol reductase cDNAs. Arrow origins indicate the 5' end of each sequencing primer. Arrow directions, left or right, indicate the strand sequenced, coding or complementary, respectively. Arrow lengths represent the amount of reliable sequence obtained per given primer.
ductase cDNA were next designed from the N-terminal (30 residues) and internal (following trypsin and cyanogen bromide digestion) amino acid sequences. With the N-terminal and reverse primers, two PCR products (380- and 400-bp from PLRN5/PLRI4R and PLRN5/PLRI5R primer pairs, respectively) were obtained as shown in Fig. 5. Verification of the authenticity of the amplified DNA was determined in two different ways. First, DNA gel blot analysis, using the 32P-end-labeled degenerate primer PLRI4R (see Fig. 4) as a probe, was performed with the amplification products of primers PLRN5/PLRI5R using purified F. intermedia cDNA library DNA as template. This gave a single band in the autoradio- graph, corresponding to the PCR-amplified band. Second, additional PCR analysis (using N-terminal and reverse primers PLRN5/PLRI4R) produced the 380-bp fragment from the 400-bp PCR species (data not shown).

The two PCR products were next cloned individually into a T-vector for restriction analysis and sequencing. Three restriction enzymes (HaeIII, Sau3A, and TaqI) were then each incubated with 12 and 24 individual inserts for the 380- and 400-bp fragments, respectively (see "Experimental Procedures"). This resulted in only one restriction group for each original PCR amplification product, thus showing that each was due to a single DNA species. Subsequent sequencing of five of the inserts (two from the 380-bp and three from the 400-bp fragments, respectively) revealed that both PCR fragments encoded the same ORF, which contained the N terminus and two of the internal amino acid microsequences of the reductase. The larger (400-bp) fragment was next radiolabeled (see "Experimental Procedures") and used, under moderately stringent conditions, to probe 600,000 pfu of the amplified F. intermedia stem cDNA library. This resulted in more than 350 positive plaques, with 20 (of different signal intensities) being subjected to two additional rounds of screening. After final purification, 8 of the 20 cDNAs encoded the desired enzyme and were subcloned by invivo excision into pBluescript. These eight cDNAs (called PLR1-PLR8) were sequenced completely with overlapping primers as shown in Fig. 6. All eight cDNAs had the same coding but different 5'-untranslated regions. On the other hand, analysis of the 3'-untranslated region of each of the eight cDNAs established that all were truncated versions of the longest cDNA's 3'-region. Preliminary RNA gel blot analysis (data not shown) with total RNA from greenhouse-grown plant stem tips confirmed a single transcript with a length of approximately 1.2 kilobase pairs.

**Fig. 7. Complete sequence of F. intermedia (+)-pinoresinol/(+)-lariciresinol reductase cDNA PLR3.** Note that the (+)-pinoresinol/(-)-lariciresinol reductase coding sequence is in frame with the α-complementation particle of the β-galactosidase gene encoded by pBluescript. Sequence data obtained by peptide microsequencing (see Fig. 4) are underlined. The NADPH binding domain conserved residues are circled. The possible N-glycosylation site (Asn-215) is indicated by a hexagon. Ser-115, conserved in all of the reductases listed under the "Results and Discussion," is indicated by triple-underlining. The five possible phosphorylation sites that are conserved among (+)-pinoresinol/(-)-lariciresinol reductase and the characterized isoflavone reductases are indicated by squares. The stop codon is indicated by an asterisk.
The amino acid composition reveals seven methionine residues. Interestingly, the N terminus of the plant-purified enzyme lacks the initial methionine, this being the most common post-translational protein modification known. Consequently, the first methionine (see Fig. 7) in the cDNA can be considered to be the site of translational initiation. The sequence analysis also reveals a possible N-glycosylation site at residue 215 (although no secretory targeting signal is present) and seven possible protein phosphorylation sites at residues 50 and 228 (protein kinase C-type), residues 228, 250, 302, and 303 (casein kinase II-type) and residue 301 (tyrosine kinase type).

In terms of identifying the regions of the polypeptide chain involved in the NAD(P)/H binding (40–42), there is a limited number of invariant amino acids in the sequences of different reductases that are viewed to be diagnostic. These include three conserved glycine residues with the sequence GXGXXG, where X is any residue, and six conserved hydrophobic residues. The glycine-rich region is viewed to play a central role in positioning the NAD(P)/H in its correct conformation. In this regard, a comparison of the N-terminal region for (+)-pinoresinol(+)-lariciresinol reductase to that of the conserved regions of Drosophila melanogaster alcohol dehydrogenase (41), Pinus taeda cinnamyl alcohol dehydrogenase (43), dogfish muscle lactate dehydrogenase (41), and human erythrocyte glutathione reductase (41) revealed some interesting parallels. As can be seen in Fig. 9, the invariant glycine residues are aligned in every case, as are four of the six hydrophobic residues required for the correct packaging in the formation of the domain. Hence, the NADPH-binding site of (+)-pinoresinol(+)-lariciresinol reductase isoforms is localized close to the N terminus (and is circled in Fig. 7).

**Homology Search: Comparison to Isoflavone Reductase**—A BLAST search (44) was next conducted with the translated amino acid sequence of (+)-pinoresinol(+)-lariciresinol reductase against the nonredundant peptide data base at the National Center for Biotechnology Information. Significant homology was noted for (+)-pinoresinol(+)-lariciresinol reductase against the phytoalexin, (+)-medioresinol(+) lariciresinol reductase and the plant protein function in precisely the same enantiospecific manner. Given that the reductase sequence is now correct, future efforts will be directed to obtaining the reductase proper heterologously expressed in order to obtain a sufficient amount for detailed biochemical analyses and crystals for X-ray analysis.

**Sequence Analysis**—The full-length sequence (see Fig. 7) of the cloned (+)-pinoresinol(+)-lariciresinol reductase (PLR3) contains all of the peptide sequences (underlined in Fig. 7) determined by Edman degradation of digest fragments (see Fig. 4). The single ORF predicts a polypeptide of 312 amino acids with a calculated molecular mass of 34.9 kDa, in close agreement with the value (~35 or ~36 kDa) estimated previously by SDS-PAGE for (+)-pinoresinol(+)-lariciresinol reductase. An equal number of acidic and basic residues are also present, with a theoretical isoelectric point (pI) of 7.09, in contrast to that experimentally obtained by chromatofocusing (pI ~ 5.7).

Fig. 8. Chiral column HPLC analysis of the lignan products obtained from the β-galactosidase- (+)-pinoresinol(+)-lariciresinol reductase fusion protein-catalyzed reaction: separation of (+)- and (−)-lariciresinols (A) and (+)- and (−)-secoisolariciresinols (B). Unlabeled (±)-lariciresinols and (±)-secoisolariciresinols were added as radiochemical carriers. The solid line represents the absorbance trace at 280 nm. Radioactivity (dashed line) is only observed in (+)-lariciresinol and (−)-secoisolariciresinol, respectively, demonstrating that the β-galactosidase- (+)-pinoresinol(+)-lariciresinol reductase fusion protein catalyzes the same enantiospecific reaction as the plant enzyme.

**Reductase**—With the cDNAs putatively encoding (+)-pinoresinol(+)-lariciresinol reductase, our next objective was to prove that the sequence was correct by heterologously expressing the enzyme in active form. (Heterologous expression is also necessary in order to obtain sufficient protein to enable the systematic study of its precise biochemical mechanism at a future date.)

Examination of the eight putative (+)-pinoresinol(+)-lariciresinol reductase clones revealed that one, PLR3, was in frame with the α-complementation particle of β-galactosidase in pBluescript (Fig. 7). This was fortuitous, since it potentially provided a facile means to express the fully functional fusion protein and, hence, to provide proof that the cloned sequence was correct. Consequently, pPLR3 was introduced into NovaBlue E. coli cells, which were grown until mid-log phase, then induced with IPTG (10 mM final concentration), grown for a further 2 h, and analyzed for expression of (+)-pinoresinol(+)-lariciresinol reductase activity.

Catalytic activity was established by incubating cell-free extracts (see “Experimental Procedures”) for 2 h at 30 °C with (±)-pinoresinols (0.4 mM) and [4R-3H]NADPH (0.8 mM). Following incubation, unlabelled (±)-lariciresinols and (±)-secoisolariciresinols were added as radiochemical carriers, with each lignan isolated by reversed-phase HPLC. As shown in Fig. 8, subsequent chiral HPLC analysis revealed that both (+)-lariciresinol and (−)-secoisolariciresinol, but not the corresponding antipodes, were radiolabeled (total activity, 54 nmol h⁻¹ mg⁻¹). By contrast, no catalytic activity was detected either in the absence of (±)-pinoresinols or when control cells were used that contained a plasmid (pPLR2) not in frame with the β-galactosidase gene. Thus, the heterologously expressed (+)-pino-
carpin (46). This sequence similarity may be more than a minor coincidence, given that both lignans and isoflavonoids are offshoots of general phenylpropanoid metabolism, with comparable plant defense functions and pharmacological roles, e.g. as phytoestrogens. Consequently, since both reductases catalyze very similar reactions, it is tempting to speculate that the isoflavone reductases may have evolved from (+)-pinoresinol/(-)-lariciresinol reductase. This is considered likely since the lignans are present in the pteridophytes, hornworts, gymnosperms, and angiosperms; hence, their pathways apparently evolved prior to the isoflavonoids.

Comparable homology was also observed with putative isoflavone reductase “homologs” from Arabidopsis thaliana (65.9% similarity, 50.8% identity), Nicotiana tabacum (48) (64.6% similarity, 47.2% identity), Solanum tuberosum (65.5% similarity, 47.7% identity), Zea mays (49) (61.6% similarity, 44.9% identity), and especially Lupinus albus (85.5% similarity, 66.2% identity). By contrast, homology with other NADPH-dependent reductases was significantly lower: for example, dihydroflavonol reductases from Petunia hybrida (50) (43.2% similarity, 21.5% identity) and Hordeum vulgare (51) (46.2% similarity, 21.1% identity), chalcone reductase from M. sativa (52) (39.5% similarity, 15.8% identity), chalcone reductase homolog from Sesbania rostrata (47.6% similarity, 24.1% identity), cholesterol dehydrogenase from Nocardi a sp. (53) (46.6% similarity, 21.0% identity), and 3-β-hydroxy-5-ene steroid dehydrogenase from Rattus norvegicus (54) (43.5% similarity, 20.6% identity).

Thus, sequence analysis establishes significant homology between (+)-pinoresinol/(-)-lariciresinol reductase, isoflavone reductases, and putative isoflavone reductase homologs that do not possess isoflavone reductase activity. Indeed, it is also interesting to note that during the purification of the eight (+)-pinoresinol/(-)-lariciresinol reductase cDNAs, two other cDNAs were purified from F. intermedia, which possess even higher levels of homology (75–83% similarity and 59–74% identity) to the putative isoflavone reductase homologs. Clearly, some other biological activity for these homologs awaits discovery, and future attention will be directed toward elucidating their function in Forsythia.

Last, with both the gene encoding (+)-pinoresinol/(-)-lariciresinol reductase and the corresponding protein now accessible, regulation of the lignan pathway and its parallels to the isoflavonoid pathway can be investigated. In this context, regulation of isoflavonoid biosynthesis is of much interest, since they are synthesized de novo in economically important crops upon exposure to elicitors from fungal pathogens. While research into the transcriptional control of isoflavone reductase has made some significant progress (see Dixon et al. (55) for a pertinent review), the mechanism of regulation of isoflavone reductase at the post-transcriptional level is unknown. It therefore seems significant that both (+)-pinoresinol/(-)-lariciresinol and isoflavone reductases contain five conserved possible phosphorylation sites, including Thr-302 (casein kinase II-type protein phosphorylase site), which are conserved in the homologs as well. Hence, it can be proposed at this point that the activities of both (+)-pinoresinol/(-)-lariciresinol and isoflavone reductases (and their transcript levels) are regulated by protein kinase cascades. This is highly likely, given the pivotal (e.g. branch-point) positions the enzymes play in their respective biosynthetic pathways, their roles in the physiology of the whole organism, and the ubiquity of protein kinase cascades in the regulation of biosynthetic pathways.

Concluding Remarks—Two isofunctional forms of (+)-pinoresinol/(-)-lariciresinol reductase were purified (>3000-fold) to apparent electrophoretic homogeneity. Both catalyze the sequential reduction of (+)-pinoresinol → (+)-lariciresinol → (−)-secoisolariciresinol and have similar kinetic parameters ($V_{max}$, $K_m$) and molecular weights. Both reductive steps proceed stereospecifically, with the incoming hydride occupying the pro-R-position at C-7 and C-7’ in both (+)-lariciresinol and (−)-secoisolariciresinol. This protein, the first isolated in the lignan pathway, is of particular interest since it catalyzes entry into the furano and dibenzylbutane lignans and is presumed to represent a regulatory point in lignan biosynthesis.

The cDNA encoding (+)-pinoresinol/(-)-lariciresinol reductase from F. intermedia has been cloned, and the recombinant protein heterologously expressed in E. coli as a catalytically active fusion protein. Its high sequence homology to isoflavone reductase may be of evolutionary significance in plant defense. Additionally, the conservation of putative protein phosphorylation sites in both (+)-pinoresinol/(-)-lariciresinol and isoflavone reductases suggests that a similar mode of regulation for both pathways may be in place. Together, these similarities suggest that although the plant defense mechanisms have been maintained, evolutionary divergence of the plant biochemical pathways has occurred. Last, since secoisolariciresinol is considered to be a major source of phytoestrogens in various high-
fiber diets, it is now clear that the systematic modulation of levels of such substances can now be investigated.

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