

**TOWARDS UNDERSTANDING THE MOLECULAR BASIS OF
ENANTIOSPECIFICITY OF PINORESINOL-LARICRESINOL REDUCTASE
OF *FORSYTHIA INTERMEDIA***



GRIFFIN CHARLES GUESS

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Dr. Norman G. Lewis

Institute of Biological Chemistry

Washington State University

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PRÉCIS

This study involves the use of an enzyme extracted from the flowering woody plant *Forsythia intermedia*. The enzyme reduces its substrate (pinoresinol) into product (secoisolariciresinol) through an intermediate (lariciresinol), and is therefore deemed a reductase of the class Pinoresinol-Lariciresinol Reductase (PLR). In question is whether a certain part of this enzyme, an amino acid, has influence over formation of its product, in particular over the spatial or stereochemical aspects of that product. Understanding the molecular basis of this process is worthwhile because these molecules, known as lignans, provide defense to vascular plants. Specifically, lignans display resistance against predation and bacterial, fungal, and viral pathogens, and contribute to plant durability during development (Katayama *et al*, 1993; Kwon *et al*, 2001). The transcendence of these benefits between plants and humans has occurred with the correlation of dietary lignans (dietary fiber) and the reduced occurrence of certain cancers. Understandably, the pharmacological potential for these plant derivatives has motivated exploration of the molecular basis of lignan biosynthesis and the foundation for this project.

The methodology used for this study included standard microbiological techniques to culture a suitable bacterial host that expressed a functionally recombinant version of the PLR enzyme in question. Multiple trial-and-error experiments were completed with the goal of optimizing enzyme expression for the most efficacious studies of the protein. Purification protocols were adopted from those used in the field of molecular biology, and procedures specific to the equipment and proteins were adapted from laboratory protocols. Assays of the enzyme were used to assess the activity of both crude and purified samples of the reductase. Site-directed mutagenesis altered the identity of the amino acid in question, in order to study the influence this position has on PLR activity. To study the stereochemical changes instigated by site-directed mutagenesis, chiral (stereochemical) chromatography was employed for separating

and quantifying the abundance of enantiomers (spatially distinct molecules) of product and substrate. This was in turn used for forming conclusions regarding the enantiospecific differences in catalysis between the native and mutant reductases.

Upon applying these methods towards the hypothesis, the study found that the area of question within the protein indeed exhibited influence over the enzyme's catalysis of substrate into product. However, the results provisionally indicate that the influence by this position is general rather than enantiospecific, with a very significant suppression of overall catalysis normally performed profusely in the native reductase. The reduced activity was also exacerbated by the lengthy experimental manipulation needed for purifying the protein. Collectively, the factors contributing to reduced overall catalysis inhibited any detailed enantiomeric or kinetic analyses of the mutated reductase. These results are similar to a separate study performed on a functionally analogous PLR from *Thuja plicata*, the western red cedar. An identical mutation of the same amino acid position in this protein yielded a reduction of overall catalysis with no apparent influence on enantiospecificity.

This study may contribute to further understanding of the reduction mechanisms and components utilized by PLRs. Although a piece of the molecular process has been tentatively uncovered, the quantitative nature and exact mechanisms for the formation of these unique molecules remain to be studied. This understanding will in turn verify the homologies between PLR-containing species, and elucidate the functional evolution of this component of plant metabolism. Further extrapolation may yield a better perspective on lignan function, synthesis, and pharmacological significance.

INTRODUCTION

Certain plant natural products, known as lignans, have arisen over the course of vascular plant evolution to provide a selective advantage against predation and infection (Kwon *et al*, 2001). The presence of these 'secondary metabolites' in woody plants has been attributed to various defensive mechanisms against bacterial, fungal, and viral pathogens, predation by insects, as well as to help confer overall durability during growth and development (Katayama *et al*, 1993; Kwon *et al*, 2001). These properties may have similar benefits in humans. For example, derivatives of the antiviral metabolite podophyllotoxin from *Podophyllum* are widely used in cancer treatment, while (-)-trachelogenin has exhibited inhibition of reverse transcriptase and thus could be important for the treatment of HIV (Diagram 1) (Katayama *et al*, 1993; Dinkova-Kostova *et al*, 1996). Similarly, the correlation between dietary fiber and the lower occurrence of certain cancers has been attributed to lignans such as the chemopreventative secoisolariciresinol diglucoside (SDG) found abundantly in flax seed (Diagram 1) (Dinkova-Kostova *et al*, 1996; Ford *et al*, 2001). It is therefore understandable that the mechanisms of lignan biosynthesis are highly sought after, as these compounds may have great potential for use in human pharmacology.

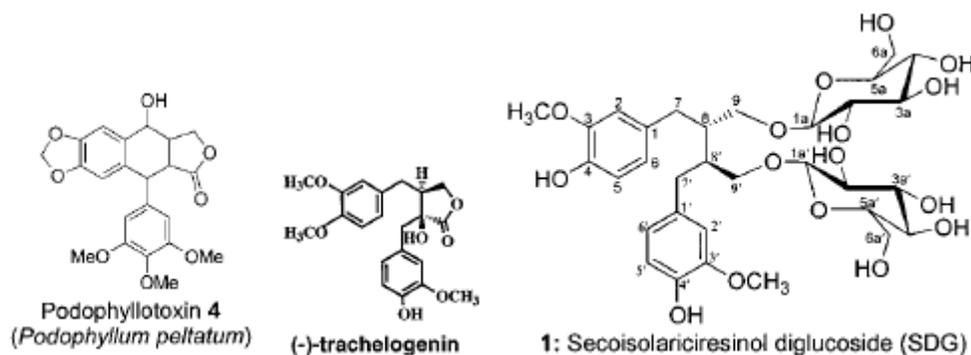


Diagram 1: Chemical structures of podophyllotoxin from *Podophyllum* (Fujita *et al*, 1999), (-)-trachelogenin (Dinkova-Kostova *et al*, 1996), and secoisolariciresinol diglucoside (SDG) (Ford *et al*, 2001)

The lignans described above come from specific species of woody plants and often represent distinct compounds in both structure and function. Fortunately, a common mechanism in the biosynthesis of these metabolites exists between several well-studied species. A stepwise reduction leading to the formation of secoisolariciresinol provides a basis for the subsequent biosynthetic steps to the compounds described above. This reaction is catalyzed by an enzyme known as Pinoresinol-Lariciresinol Reductase (PLR), aptly named for the two substrates it reduces (Diagrams 2 and 3). PLRs are generally capable of discriminating between specific enantiomers of substrate and subsequently reducing them to product. For example, a PLR for *Forsythia intermedia* (PLR_Fi1) catalyzes the enantiospecific reduction of (+) pinoresinol to (-) secoisolariciresinol (Dinkova-Kostova *et al*, 1996). This enantiospecificity, however, can vary between species as does the stereochemistry of the product, secoisolariciresinol.

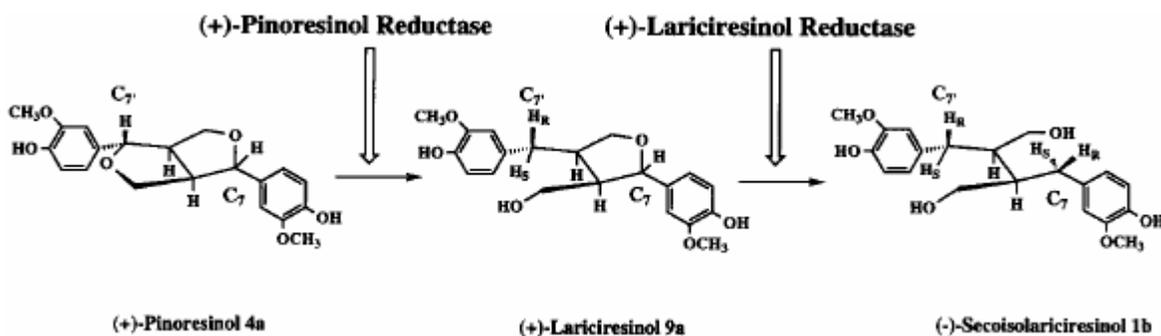


Diagram 2: Reductions catalyzed by PLR_Fi1 (Chu *et al*, 1993) and PLR_Tp2 (Fujita *et al*, 1999)

For example, western red cedar *Thuja plicata* contains two PLR-like enzymes. The first contains a PLR enzyme (PLR_Tp1) that catalyzes the same reaction albeit using (-) pinoresinol preferentially; however, it has the capability to less efficiently reduce the (+) pinoresinol antipode into (+) lariciresinol (Fujita *et al*, 1999). *T. plicata* also possesses a second PLR (Tp2) that catalyzes an opposite enantiomeric reduction from (+) pinoresinol to (-) secoisolariciresinol, with the formation of both lariciresinol antipodes (Fujita *et al*, 1999) (Diagram 2). However, as

for PLR_Tp1, it can also less efficiently utilize the (-) antipode of pinoresinol. Additionally, the precursor needed for SDG, for instance, is mainly the (+) form of secoisolariciresinol, and the plant that creates this product, *Linum usitatissimum*, contains a reductase (PLR_Lu1) that mainly reduces (-) pinoresinol as shown in Diagram 3.

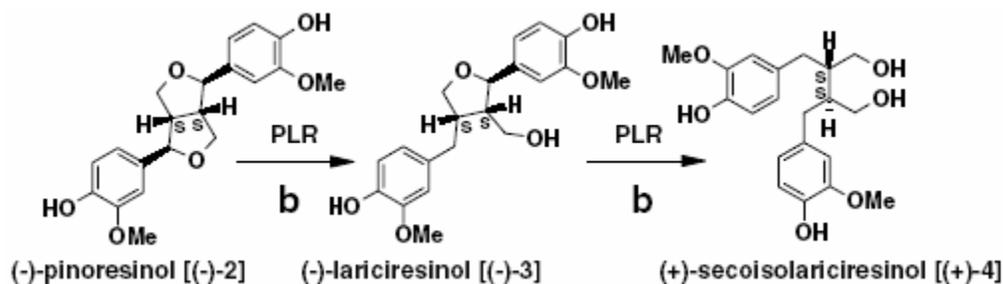


Diagram 3: Progression of reduction by PLR_Tp1 (Fujita *et al*, 1999) and PLR_Lu1 (Heimendahl *et al*, 2005)

These studies demonstrate that different plant species can have differing reductive processes, in terms of the overall enantiospecificity, to yield a particular product of importance to the plant. Identifying the molecular basis of these enantiospecific mechanisms would thus be advantageous for understanding and manipulating the synthesis of pharmacologically important compounds from flax and *Podophyllum*, for instance. In this regard, PLRs from *T. plicata* have provided a great deal of published information on these interesting enantiospecific reactions, particularly with regards to detailed kinetic data and x-ray crystallographic analysis. Min *et al* (2003) in particular provided a spatial analysis of PLR_Tp1 and Tp2, and identified homology between the two enzymes in the primary sequence and functional quaternary structures of the proteins. Three particular amino acids were identified in the binding pocket of the enzyme that potentially dictated the molecular basis of the enantiospecific differences between Tp1 and Tp2. In the case of Tp1, Phe¹⁶⁴, Val²⁶⁸, and Leu²⁷² were considered to favor accommodation of (-) pinoresinol, whereas Leu¹⁶⁴, Gly²⁶⁸, and Phe²⁷² in Tp2 favors binding of (+) pinoresinol (Min *et*

al, 2003). That is, one possibility was that the enantiospecific differences were based on the symmetrical amino acid substitutions as above in the (+) and (-)-forming PLRs.

As indicated above, the PLR_Fi1 catalyzes a reaction comparable to Tp2, in which (+) pinoresinol is enantiospecifically converted to (+) lariciresinol and then to (-) secoisolariciresinol (Katayama *et al*, 1993; Dinkova-Kostova *et al*, 1996). The first step is exclusively preferential for the (+) form of pinoresinol, and the second reduction strictly occurs with (+) lariciresinol (Katayama *et al*, 1993). The kinetics and enantiospecificity of this reaction are well studied in this species, although the molecular basis for the enantiomeric specificity has not been investigated. This project investigated the effect, if any, of targeted site-directed mutagenesis on prospective amino acids potentially controlling the enantiospecificity.

Hypothesis and Objective

In this study, we hypothesized that using a site-directed mutagenesis approach, to mutate the neutral amino acid Gly²⁶⁸ in the PLR_Fi1 sequence into the corresponding hydrophobic amino acid Val²⁶⁸, would establish whether or not this residue was directly involved in determination of enantiospecificity.

EXPERIMENTAL METHODOLOGY

Gene Isolation & Sequencing – Three plasmid preparations were available from Dr. David Gang's previous work, each possibly containing the PLR *Forsythia intermedia* gene: Stratagene[®]'s pBluescript SK (+) (pBS), Novagen[®]'s pSBET and pETBlue. Each preparation was sequenced for congruency with the Fi1 gene sequence (Figure 1).

```
1 aaaaacagag agagatggga aaaagcaaag ttttgatcat tgggggtaca ggggtacttag
61 ggaggagatt ggtaaggca agtttagctc aaggatcatga aacatacatt ctgcataggc
121 ctgaaattgg tgttgatatt gataaagttg aaatgcta atcattttaa atgcaaggag
181 ctcatcttgt atctggttct ttcaaggatt tcaacagtct ggtcaggct gtcaagctcg
241 tagacgtagt aatcagcgcc atttctgggtg ttcatattcg aagccatcaa attcttcttc
301 aactcaagct tgttgaagct attaaagagg ctggaaatgt caagagattt ttaccatctg
361 agtttggaat ggatcctgca aaatttatgg atacggccat ggaacccgga aaggtaaacac
421 ttgatgagaa gatggtggta aggaaagcaa ttgaaaaggc tgggattcct ttcacatatg
481 tctctgcaaa ttgctttgct ggttatttct tgggaggctct ctgtcaattt ggcaaaattc
541 ttccttctag agattttgtc attatacatg gagatggtaa caaaaaagca atatataaca
601 atgaagatga tatagcaact tatgccatca aaacaattaa tgatccaaga accctcaaca
661 agacaatcta cattagtctt ccaaaaaaca tcctttcaca aagagaagtt gttcagacat
721 gggagaagct tattgggaaa gaactgcaga aaattacact ctogaaggaa gattttttag
781 cctcctgtaa agagctcgag tatgctcagc aagtgggatt aagccattat catgatgtca
841 actatcaggg atgccttacg agttttgaga taggagatga agaagaggca tctaaacttt
901 atccagaggt taagtatacc agtgtggaag agtacctcaa gcgttacgtg tagttgaaag
961 ctttccatta ttattgtaat aatattttaa tcagtatgta gtttttaatt tegttaaata
1021 atatgtggtg aattttgctt ccaaaaa
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MGKSKVLIIGGTGYLGRRLVKASLAQGHETYILHRPEIGVDIDK
VEMLISFKMQGAHLVSGSFKDFNSLVEAVKLVVVISAISGVHIRSHQILLQLKLVFA
IKEAGNVKRFLPSEFGMDPAKFMDTAMEPGKVTLDEKMWVRKAIEKAGIPFTYVSANC
FAGYFLGGLCQFGKILPSRDFVLIHGDNKKAIYNNEDDIATYAIAKTINDPRTLNKTI
YISPPKNILSQREVVQTEWKLIGKELQKITLSKEDFLASVKELEYAQQVGLSHYHDVN
YQGCLTSFEIGDEEEASKLYPEVKYTSVEEYLKRYV
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Figure 1: PLR_Fi1 gene sequence & protein translation (NCBI.org)

In each case, a 10- μ L polymerase chain reaction (PCR) was performed using *Taq* polymerase with the appropriate primers as follows: forward direction with the T7 promoter primer, and in the reverse direction with the T3 primer for pBS, as well as the custom FiSEQ primer for pSBET and pETBlue (Table 1). Samples were inserted into a Peltier thermal cycler for the sequencing reaction (24 cycles of 20 sec at 96° C, 30 sec at 50° C, and 3 minutes at 60° C), filtered using Edge BioSystems[®] Performa[®] Gel Filtration Columns, vacuum dried, and sequenced at the Washington State University Sequencing Laboratory.

DNA Primer	Sequence (5 - 3)	Locality
T7 FORWARD	TAATACGACTCACTATAG	pBS
T3 REVERSE	CCTTTAGTGAGGGTTAATT	pBS
PLR_Fi1-FOR-MUT	GAGTATGCTCAGCAAGTGGTGTTAAGCCATTATCATGATG	SDM
PLR_Fi1-REV-MUT	CATCATGATAATGGCTTAACACCACTTGCTGAGCATACTC	SDM
PLR_Fi1-REVTAG	CTACACGTAACGCTTGAGGTAC	PLR_Fi1
Fi-SEQ	ACCCGGAAAGGTAACACTTG	pSBET/ pETBLUE

Table 1: Primer sequences

The chosen pBS plasmid was transformed into Invitrogen[®]'s Top10 One Shot Chemically Competent *Escherichia coli* cells following the manufacturer's instructions, and cells were incubated overnight at 37° C on Luria-Bertani (LB) plates supplanted with 100 µg/mL carbenicillin. Cells were screened with a 20 µL PCR (40 cycles of 30 sec at 96° C, 30 sec at 55° C, 2 min at 72° C with a final annealing time of 10 min at 72° C) containing Promega's *Taq* polymerase and 10x buffer, 25 mM MgCl₂, 2 mM each dNTP, and T3 and REVTAG primers (Table 1). The amplified product was subjected to electrophoresis at 50 mA on an agarose (1%) gel containing ethidium bromide. The final gel was visualized under UV light and colonies containing the correct segment of DNA were selected. Plasmid preparations were made following Promega's Wizard[®] Plus SV Minipreps DNA Purification System instructions.

Cell Cultures and PLR_Fi1 Protein Harvests – A preliminary small-scale purification of PLR_Fi1 was performed with the pBS/TOP10 combination to test for induction fidelity and crude expression of the protein. Correctly transformed *E. coli* cells were inoculated into two tubes of 5 mL LB broth supplanted with 100 µg/mL carbenicillin and incubated at 37° C. Growth readings were taken using a spectrophotometer at an absorbance of 600 nm and the culture was grown to mid-log phase OD₆₀₀ = ~0.7. Cells were collected by centrifugation (1000 x g 10 min) and resuspended in LB broth, one tube induced with 1 mM isopropyl-β-D-

thiogalactopyranoside (IPTG) and the control tube without. Both sets of cells were grown overnight at 37° C and collected by centrifugation (1000 x g 10 min). Cell pellets were frozen at -80° C for one hour and resuspended in buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 5 mM dithiothreitol), then sonicated (3 x 30 s) and again centrifuged (17,000 x g 10 min).

Polyacrylamide Gel Electrophoresis (PAGE) –Soluble protein (4 µg) was mixed with loading buffer and dithiothreitol to a final volume of 20 µL. Samples were denatured by boiling and loaded onto a 4-15% Tris-HCl polyacrylamide gel for PAGE at 25 mA, with the resulting gel stained with silver (30 min fixation ethanol/glacial acetic acid, 30 min sensitization ethanol/sodium thiosulfate/sodium acetate, 15 min H₂O wash, 20 min silver stain 1% silver nitrate/0.04% formaldehyde, 5 min H₂O wash, develop 3% sodium carbonate/0.04% formaldehyde, 10 min stop EDTA, 30 min preserve ethanol/glycerol).

Large-Scale Culture– A large scale culture of cells was performed for PLR_Fi1 protein purification. The culturing scheme was followed as before, using 6 x 1L flasks of 250 mL media for a total volume of 1.5 L, grown to OD₆₀₀ = ~0.7. After centrifugation (1000 x g 10 min), portions were induced and grown at 23° C for approximately 20 hours. Resulting cells were centrifuged (5,000 x g 20 min), frozen at -80° C for one hour, and resuspended in 10 mL buffer (20 mM bis-Tris propane, 2 mM EDTA, 5 mM dithiothreitol, pH 8.0) for sonication (3 x 30 s) and centrifuged in a fixed angle rotor (25,000 x g 30 min). Crude protein supernatant was precipitated with ammonium sulfate [(NH₄)₂SO₄] between 20% and 50% saturation, and the resulting centrifuged (25,000 x g 30 min) pellet was frozen at -20° C. Pellets were dissolved in the same buffer and desalted over a PD-10 column.

PLR Assays – Enzyme assays consisted of a 250-µL reaction containing 20 µL 5 mM (±)-pinoresinols, 20 mM Tris-HCl pH 8.0 to volume, and up to 210 µL of protein of varying

concentration. The reaction was initiated with 20 μ L 10 mM NADPH and conducted at 30° C for 30 minutes. The reaction substrates/product were extracted with ethyl acetate (2 x 250 μ L) and desiccated in a vacuum dryer.

Site-Directed Mutagenesis –The PLR_Fi1 gene was mutated using Stratagene[®]'s Quikchange XL Site-Directed Mutagenesis kit and custom designed DNA primers PLR_Fi1-FOR-MUT and PLR_Fi1-REV-MUT (Table 1). The manufacturer's PCR protocol (18 cycles of 50 sec at 95° C, 50 sec at 60° C, 4 min at 68° C with a final annealing of 7 min at 68° C) was followed in order to replicate mutant DNA containing a valine at position 268 in the PLR_Fi1 gene. DNA was tested with an agarose gel screen, and subsequently transformed into XL-10 Gold *E. coli* cells. Cells were grown overnight and colony plasmids were prepped and sequenced. Mutant plasmids containing the appropriately mutated Gly268Val_PLR_Fi1 gene were transformed into TOP10 cells.

PLR_Fi1 Purification (General Procedures)

AffiBlue Affinity Purification Column – An affinity column was equilibrated in buffer A pH 8.0 (20 mM bis-Tris propane, 2 mM EDTA, 5 mM dithiothreitol) in a Pharmacia FPLC overnight to achieve a stable baseline (flow rate 1 mL•min⁻¹). Samples were next loaded onto this column and eluted using buffer A and a pre-stored gradient program (5.5 mL injection volume, flow rate 1 mL•min⁻¹, 4 mL fractions x 70; gradient 0 M NaCl to 1.25 M NaCl in 120 mL). The fractions containing the greatest amounts of PLR (estimated by PAGE analysis) were concentrated using a Centricon[®] Plus centrifugal filter, desalted over a PD-10 column and used immediately for further purification.

Poros 20 HQ (1.662 mL) Anion Exchange Column Chromatography – This was performed on a Poros 20 HQ column (using a BioCAD Perfusion Chromatography Workstation

by Perceptive Biosystems) and eluted with buffer A (pH 8.0) as for the AffiBlue step above (3 mL injection volume, flow rate 10.0 mL•min⁻¹, with gradient of 0 M NaCl to 2 M NaCl in 86 mL).

Agarose ADP Anion Exchange Column Chromatography – The final stage of PLR_Fi1 purification was performed on the BioCAD using an agarose ADP anion exchange column eluted with buffer A (pH 7.0) with a similar program (injection volume 3.7 mL, flow rate 3.0 mL•min⁻¹) and a gradient of 0 M NaCl to 2.5 M NaCl in 30 mL. The eluent from this step gave apparently homogenous PLR_Fi1 for enzyme assays as needed.

Reversed-Phase High Performance Liquid Chromatography (HPLC) – A solution of (±)-lariciresinols, (±)-secoisolariciresinols, and (±)-pinoresinols in a 1:1:1 ratio was prepared for reversed-phase HPLC analysis, with an aliquot (10 µL) injected into a Waters Millennium HPLC equipped with a Novapak C₁₈ column (flow rate 0.6 mL•min⁻¹ with detection at 280 nm) and eluted with an isocratic solvent system of 70% acetic acid : 30% methanol. The same conditions were employed for all PLR assay mixtures, injecting an 80 µL aliquot for samples.

Chiral HPLC – The PLR substrate pinoresinol and PLR reduction products, lariciresinol and secoisolariciresinol, were individually collected during the reversed-phase HPLC as above and freeze-dried overnight. Lignan samples were then individually resuspended in ethanol and injected onto Chiralcel OD/OC columns using an Alliance HPLC for separation of individual enantiomers with detection at 280 nm [Chiralcel OD column (±)-pinoresinols, eluent solvents ethanol: hexanes (3:7), flow rate 0.5 mL•min⁻¹; Chiralcel OD column (±)-secoisolariciresinols, eluent solvents ethanol: hexanes (1:1) flow rate 0.8 mL•min⁻¹; Chiralcel OC column (±)-lariciresinols, eluent solvents ethanol: hexanes (4:1) flow rate 0.5 mL•min⁻¹].

RESULTS & DISCUSSION

Sequence Verification of PLR_Fi1

Initially, DNA sequencing was used diagnostically to verify that the construct contained the PLR_Fi1 gene in each of the three plasmid preparations. The sequences were returned electronically and then analyzed using BioEdit[®] software to compare with the initial PLR_Fi1 DNA sequence deposited in the National Center for Biotechnology Information (NCBI). Matching constructs to the authentic PLR_Fi1 gene is necessary to determine if there are base mismatches, as well as for showing direction in relation to the primer. Figure 2 demonstrates the utility by juxtaposing the authentic *F. intermedia* nucleotide sequence from NCBI with the sample pBluescript sequence. Sequence A was obtained using the forward primer, yet the 3' end including the stop codon TAG was sequenced, indicating that the gene was inserted backwards into the plasmid. This was substantiated further by sequence B, as the reverse primer sequenced the 5' ATG start codon end. Despite the insertion backwards, the pBS vector was chosen over the psBET and pETBlue for its relative stability and storage capability, easily available primers, selectivity by carbenicillin, and most importantly for the inducible expression of the desired gene.

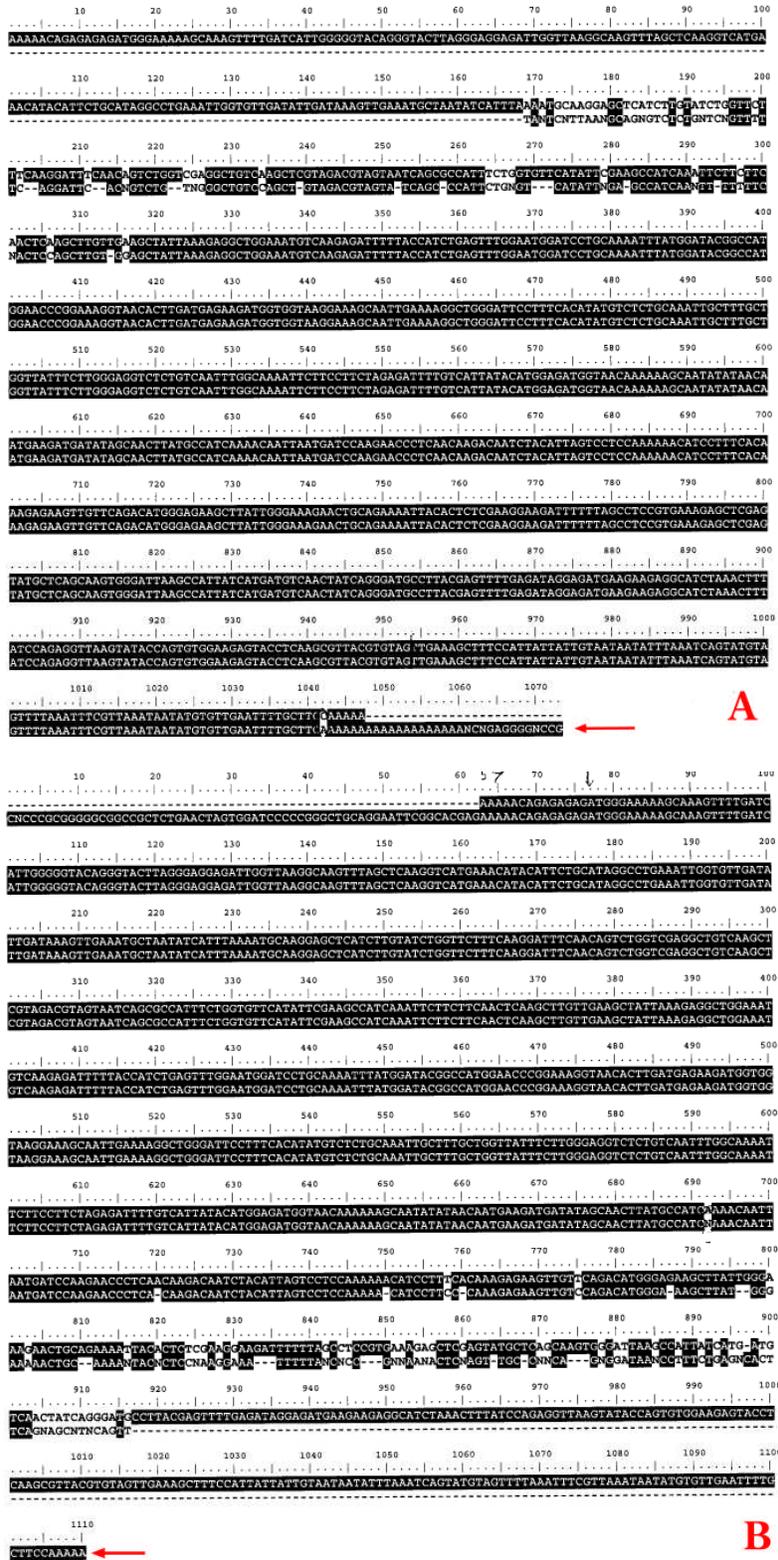


Figure 2: Sequence A primed with T7FOR and B with T3REV (Table 1). NCBI_PLR_Fi1 gene upper line, PLR_Fi1/pBS sample (red arrows) lower line

PLR_Fi1 Recombinant Protein Expression:

After similar experimentation with bacterial hosts, *E. coli* TOP10 cells were chosen as the host strain. Although the protein yield per unit of culture was relatively small, and TOP10 cells are typically used for storage purposes, the pBS/TOP10 combination nevertheless provided active, soluble, PLR_Fi1 protein for use in this project. Most importantly, the PLR protein could be expressed in its native, folded, form without any indication of toxicity to the host. In addition, the target protein was expressed into the cell's cytoplasm without formation of lipid inclusion bodies that would prevent facile purification of the protein. Cells were easily lysed with a sonicating probe in the liquid culture as previously described, breaking open the hydrophobic bilayer of the cell wall thereby affording soluble protein for further use.

All of these reasons made the PLR_Fi1/pBS/TOP10 combination advantageous, especially considering the lack of success attaining soluble and active protein from other cell combinations (data not shown). The only drawback again was the relatively low PLR_Fi1 protein yield, with this being ameliorated by use of larger cell culture volumes to optimize production.

Crude PLR_Fi1 Assays: Verification of PLR Activity

With both PLR_Fi1 gene expression and the recombinant protein expression in place, preliminary experiments using crude protein extracts were carried out to establish the presence of functionally competent PLR_Fi1.

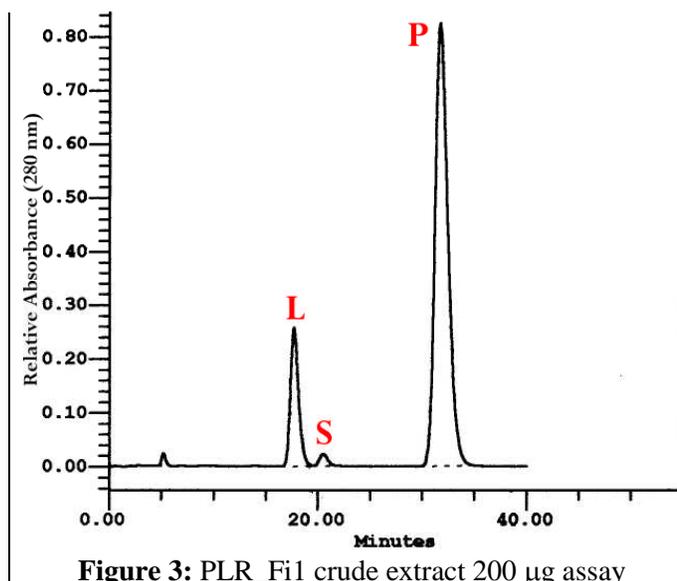


Figure 3: PLR_Fi1 crude extract 200 µg assay

Preliminary harvests and assays next demonstrated that 200 µg of soluble, crude, PLR_Fi1 protein was able to catalyze the reduction of pinoresinols (P) into the products lariciresinols (L) and secoisolariciresinols (S) (Figure 3). As shown, lariciresinol is eluted first at a retention time of 17.7 minutes, followed immediately by secoisolariciresinol at 20.5 minutes. The substrate, pinoresinol, follows as the largest peak, eluting between 29.8 and 35.1 minutes. Identification of these retention times had previously been established by running a standard 1:1:1 ratio of all three molecules prior to the assays. Sample times were thus compared between the authentic standards and the assay mixtures, all being within expected experimental variation; minor differences in elution volume result from slightly different column chromatographic eluent conditions from day to day that lead to slight retention time disparities. Further verification of the identity of the assay mixtures containing lariciresinol, secoisolariciresinol, and pinoresinol required spectroscopic analysis of each of the peaks present in the HPLC chromatogram. Figure 4 shows the characteristic UV absorbance spectrum for the lariciresinol (L) and secoisolariciresinol (S) used and formed by this reductase.

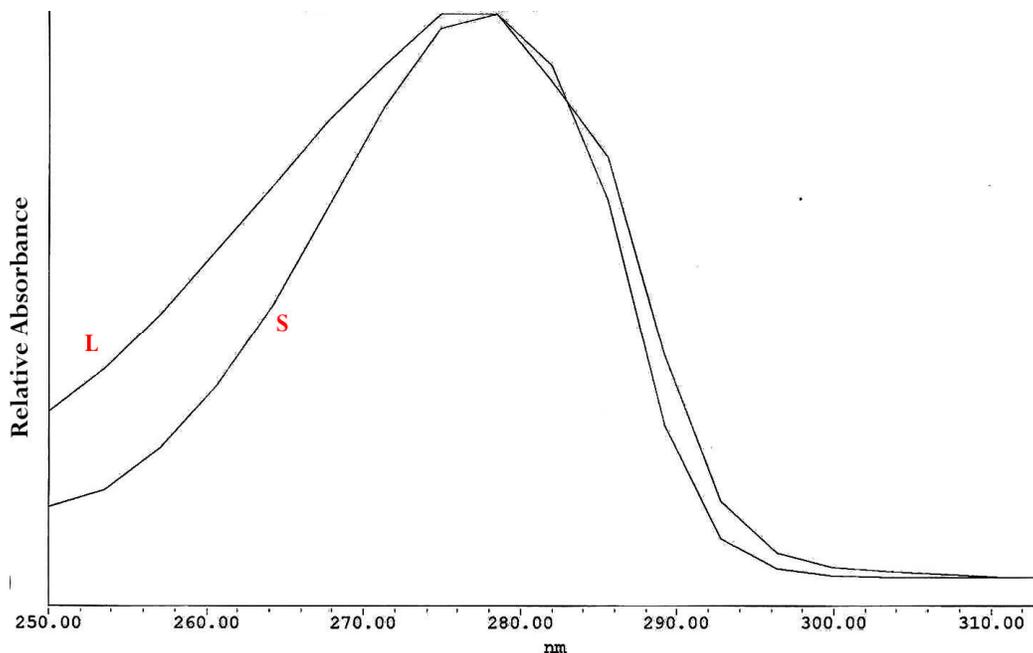


Figure 4: HPLC spectrum of lariciresinol (L) and secoisolariciresinol (S)

The absorbance spectrums of the sample peaks were identical to the authentic standards, thereby indicating that a reduction to afford both lariciresinol and secoisolariciresinol had occurred. For further control purposes, using the above methods, an assay for an un-induced control was also carried out. In the absence of IPTG, there was little to no lariciresinol or secoisolariciresinol formed, indicating that this reduction required PLR_Fi1 recombinant protein induction and that a negligible amount of basal activity occurs for this construct and host combination (Figure 5).

There was no detailed enzyme kinetic data taken for this project. It was assumed that using a greater amount of protein would yield a higher conversion of pinoresinol into secoisolariciresinol in a Michaelis-Menten fashion. The remaining variable to test, therefore, was the amount of active, soluble reductase in each sample assayed. Figure 6A depicts a chromatogram for an assay

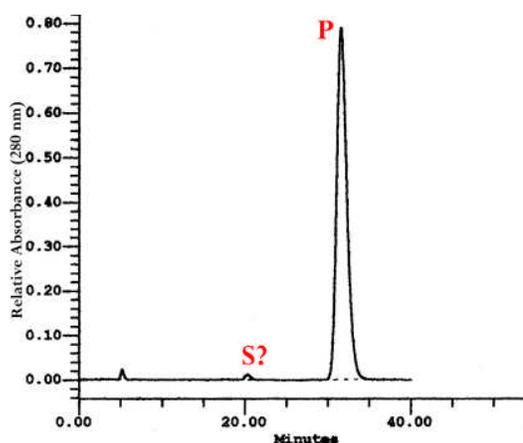


Figure 5: 100 μ g assay of PLR_Fi1 recombinant native protein from un-induced host cells

containing active reductase. It is evident that the substrate was converted into products at an appreciable amount, as opposed to the weakly active sample shown in Figure 6B.

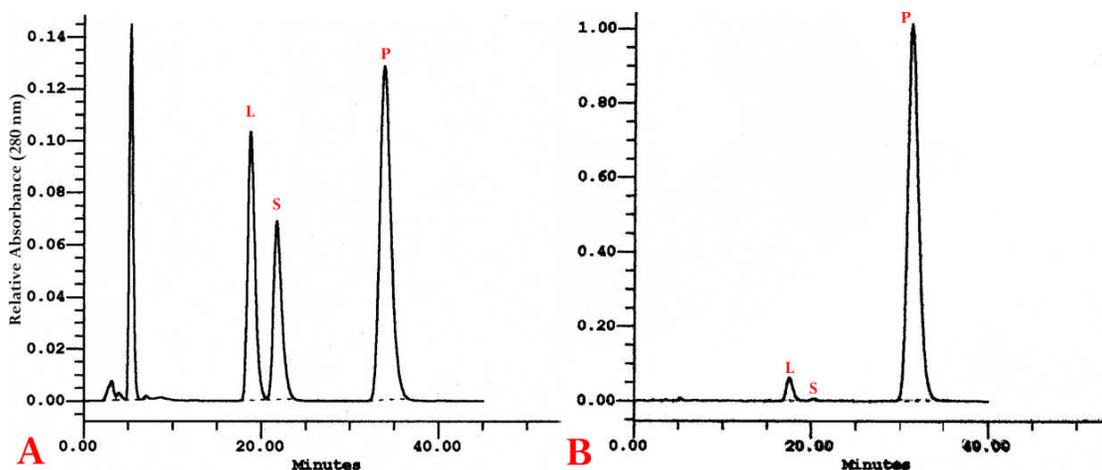


Figure 6: Disparity in activity between two separate samples of recombinant native PLR_Fi1; (A) appreciably active and (B) weakly active

Knowing that the PLR_Fi1 enzyme in a crude extract was active, attention was moved to the enantiospecificity of the PLR_Fi1 catalyzed reaction. To do this, the products lariciresinol (L) and secoisolariciresinol (S), as well as the substrate pinoresinol (P) were each isolated by reversed-phase HPLC and then individually subjected to chiral HPLC separation. Again, standards for each compound were run concurrently with the samples, allowing for a control to reference unknown retention times. The following chiral chromatograms depict the absorbance of a particular product or substrate, each peak representing the relative amount of pure enantiomers. These chromatograms are best interpreted using the percent area beneath each peak and, unlike the Millennium HPLC, the software for this particular machine allows for manual selection of individual peaks for processing areas and relative percentages. This isolates the targeted peaks for more accurate interpretation of enantiomer amounts. The standards are 1:1 racemic mixtures of both antipodes, and the percent areas are therefore approximately 50%. Enantiospecificity is indicated by a shift in the ratio of these relative percentages, and preference for one enantiomer or another can be determined. Figure 7 below illustrates the chiral separation

of racemic lariciresinol enantiomers, showing the standard retention times for elution and the calculated areas and percent areas of each peak.

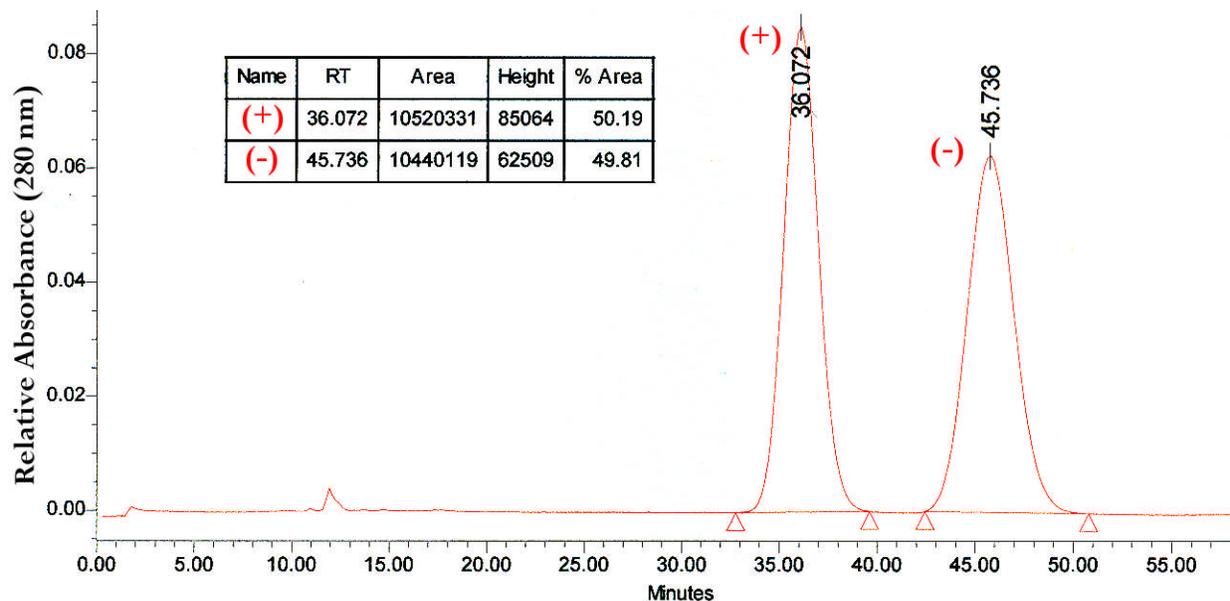


Figure 7: Lariciresinol chiral column HPLC standard injection

In contrast, the lariciresinol formed enzymatically by PLR_Fi1 (Figure 8) only includes the exclusive presence of the (+) antipode.

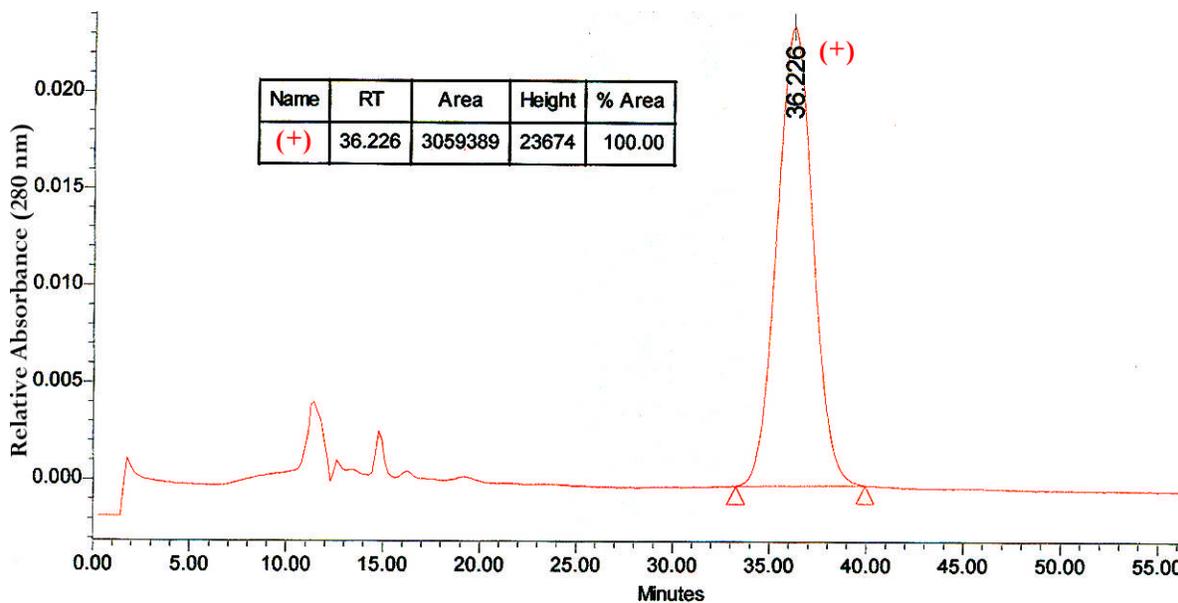


Figure 8: Lariciresinol chiral HPLC from 50- μ g assay of crude native PLR_Fi1

Similarly, chiral separation of the secoisolariciresinol enantiomers displays an elution profile shown in Figure 9 below.

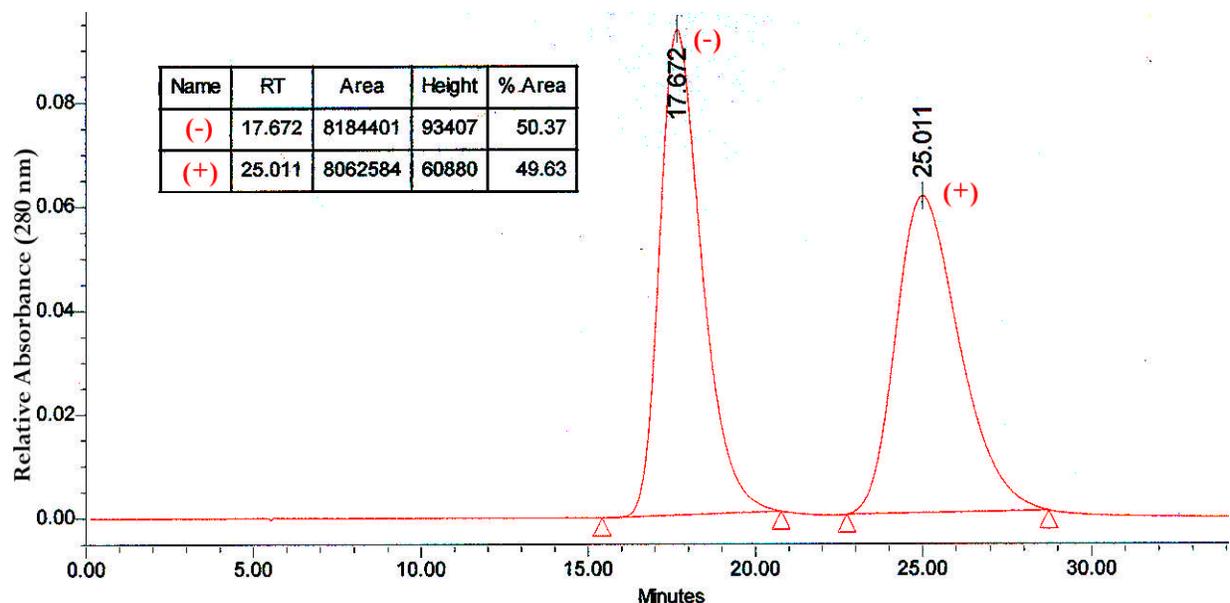


Figure 9: Secoisolariciresinol chiral column HPLC standard injection

The amount of secoisolariciresinol formed by PLR_Fi1 in this particular instance was minute in comparison to the standard peaks (Figures 9 and 10). Nonetheless, a peak was tentatively identified corresponding to the (-) antipode, with no apparent presence of another enantiomer.

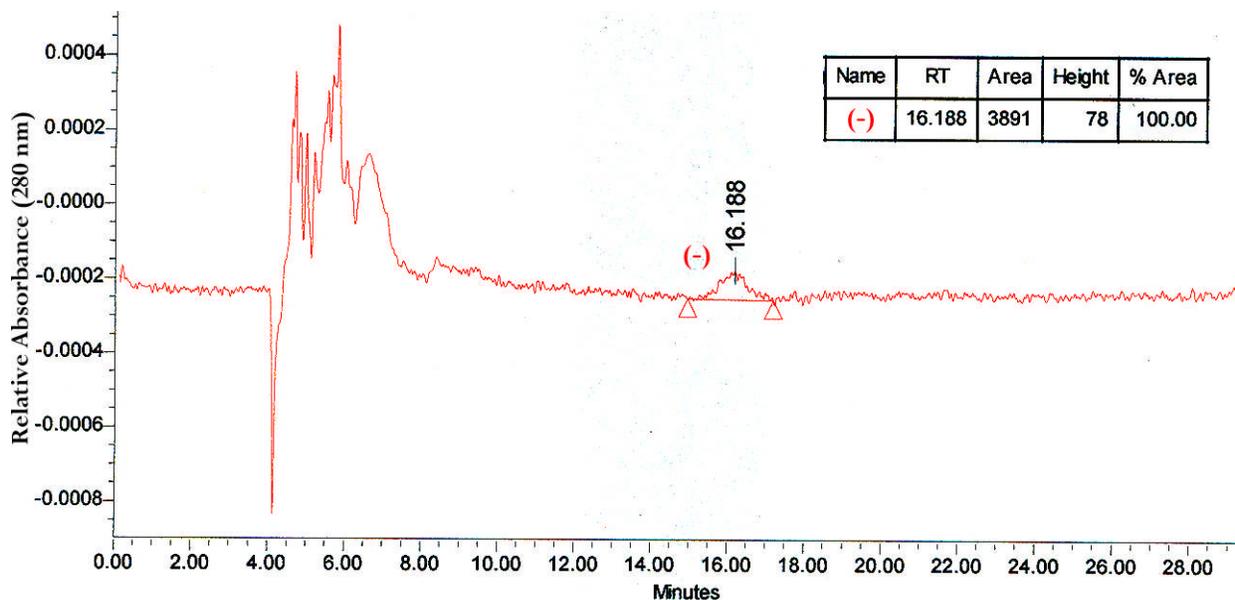


Figure 10: Secoisolariciresinol chiral HPLC from 50- μ g assay of crude native PLR_Fi1

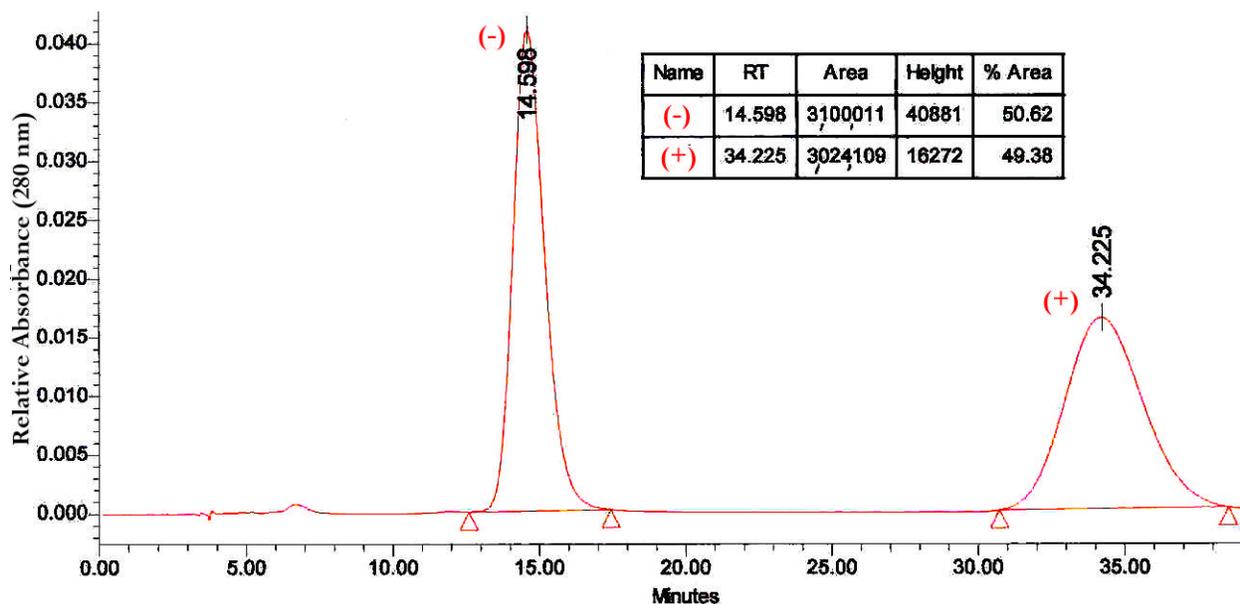


Figure 11: Pinoresinol chiral column HPLC standard injection

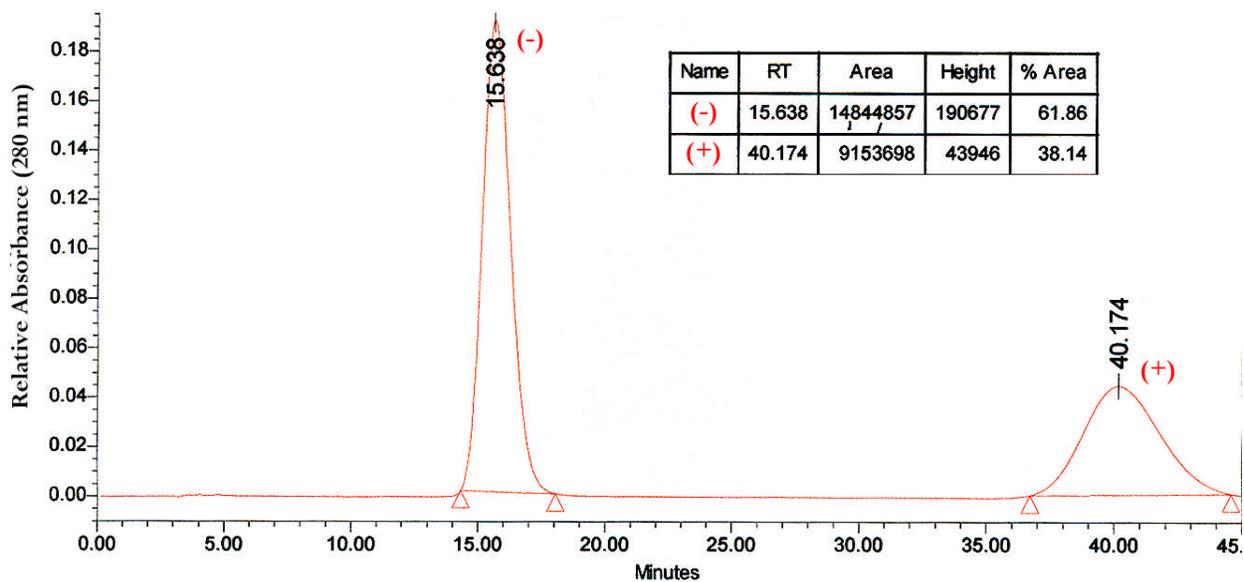


Figure 12: Pinoresinol chiral HPLC from 200- μ g assay of crude native PLR_Fi1

Lastly, the pinoresinol used by PLR_Fi1 as a substrate for the formation of lariciresinol and secoisolariciresinol displayed an enantiospecific preference for reducing the (+) antipode, as shown between the standard injection (Figure 11) and the enzymatic sample (Figure 12).

The results of the chiral analyses of these three participants support the idea that PLR_Fi1 indeed catalyzes an enantiospecific reduction of substrate to product, favoring the use of (+) pinoresinol and ultimately forming (-) secoisolariciresinol through a (+) lariciresinol intermediate.

PLR_Fi1 Purification to Apparent Homogeneity

While the above indicates that functionally competent PLR_Fi1 was being expressed, attention was next geared towards purification to apparent homogeneity. In this regard, PAGE was used to monitor the progression of the various purification steps through visual analysis of developed gels. A ladder protein was included in each gel and juxtaposed against the samples for referencing sizes of bands. PLR_Fi1 is a 34.9 kDa protein (Dinkova-Kostova *et al*, 1996) that appears on a gel between the 30 kDa and 40 kDa ladder bands, and is noted below in Figure 13 by the red arrow.

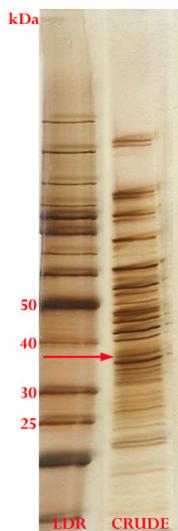


Figure 13: Native PAGE noting PLR_Fi1

The first step in removing a portion of the extraneous bands shown above and below PLR_Fi1 in Figure 13 was ammonium sulfate precipitation. This salt was systematically added to the crude protein at calculated increments to sequester certain proteins out of solution and into a collectable pellet of insoluble material. After centrifugation, the supernatant containing the remaining soluble protein components could be removed and subjected to the same process at a higher osmolarity. Repetition of this process resulted in a final pellet of protein representing a specific range of ammonium sulfate percentages, with all proteins that

precipitated outside of that range excluded from this pellet. Experimenting to find the most appropriate segment of ammonium sulfate precipitation for PLR_Fi1 showed that a pellet

between 20-50% encompassed the targeted reductase. Although PAGE analysis was performed for these experiments (data not shown), an enzymatic assay of each increment was more helpful. Activity was determined with a brief assay and subsequent HPLC analysis of both the supernatant and the pellet for each 10% increment, starting at 20% and ending at 60% ammonium sulfate. Although the established, most successful, gradient for PLRs according to Dr. David Gang's laboratory protocol is a 70% pellet from a 40% initial concentration, PLR_Fi1 displayed substantial activity from a 20% pellet in this study (Figure 14A). By contrast, the 50% supernatant catalyzed very little substrate into products indicating that most of the PLR had precipitated by this point (Figure 14B).

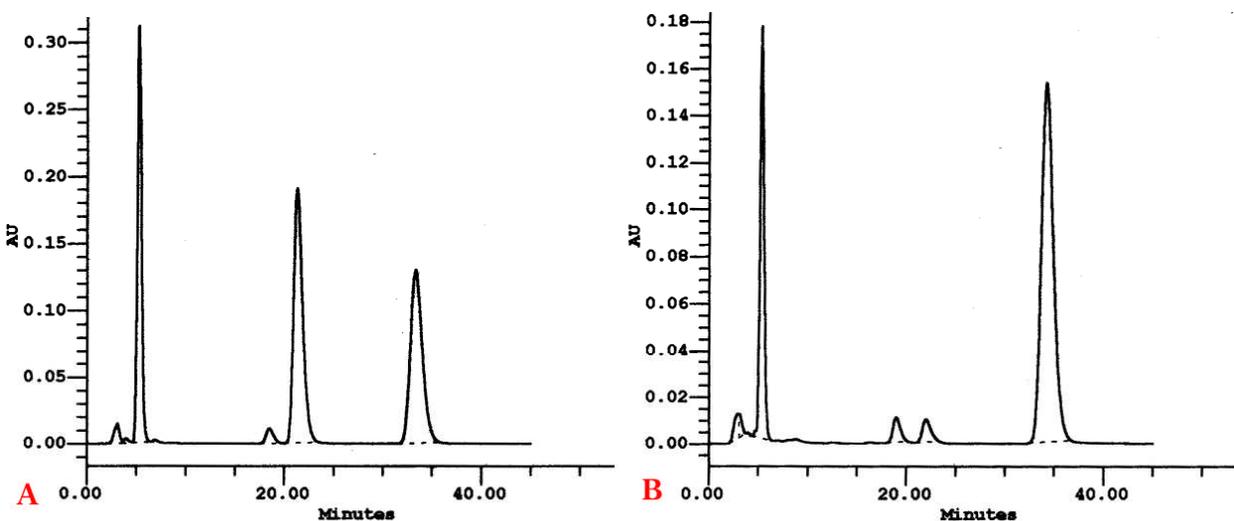


Figure 14: Brief assays (unknown concentrations) of native PLR_Fi1 samples from (A) 20% $[(\text{NH}_4)_2\text{SO}_4]$ pellet and (B) 50% $[(\text{NH}_4)_2\text{SO}_4]$ supernatant

Next, affinity column chromatography was investigated by taking advantage of the specific ionic characteristics of differing proteins and their respective differential affinities for an AffiBlue column. Just as in the ammonium sulfate stage, a gradient of ions instigates a change in the ionic character of the proteins. In this case, an automated FPLC integrated a linear, continuously increasing gradient of sodium chloride into the affinity column elution buffer (see

Experimental Methodology), causing the systematic elution of the column-bound proteins. The elution was monitored by UV spectrometry and graphed against both time and the NaCl gradient in the buffer. The resulting chromatogram (Figure 15) includes notation of each 4 mL fraction collected from the column, whereby PLR_Fi1 eluted during the latter 1/3 of the gradient, i.e. in the area between the arrows, as evidenced by PAGE analysis. Figure 16 (see red arrow) shows that the reductase was mainly present from fractions 34 to 47, diminishing by fraction 49.

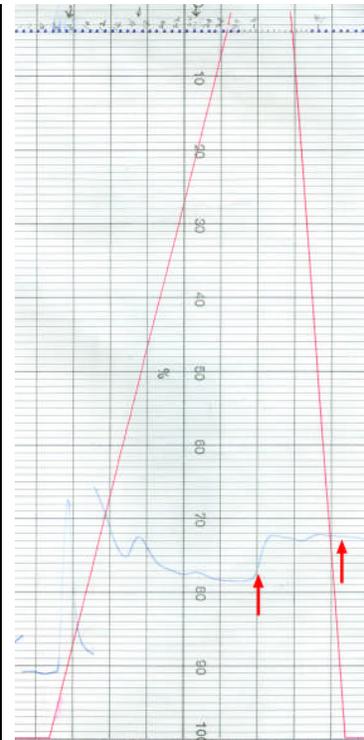


Figure 15: Native PLR_Fi1 AffiBlue affinity column purification chromatogram

By this stage of purification, the gel band corresponding to PLR_Fi1 was considerably more intense than in the crude sample, indicating that purification had progressed as planned. The fractions illustrated were next concentrated and further purified by application to an HQ column. Once again, an ionic gradient segregated proteins as they eluted into fractions, whereby a similar separation process ensued. The sample had been purified considerably by this point, although an additional *E. coli* band was present at approximately 40 kDa (data not shown).

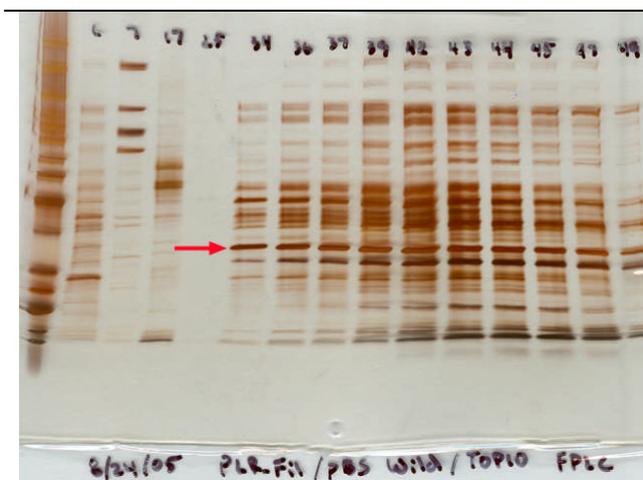


Figure 16: PAGE of native PLR_Fi1 AffiBlue fractions

This unwanted band required use of an additional purification step with an ADP column. The process for this step was similar and performed on the same machine as the HQ, although an agarose gel column was used that successfully removed the 40 kDa

band. The progression of this entire process is depicted in the following succession of gels as Figure 17, for each of the chromatographic stages.

Establishing an adequate protocol for purifying the native protein also functioned as a form of control when the time came to repeat the process for the mutant. The goal for the purification scheme was to obtain recombinant PLR_Fi1 protein of at least 90% purity. This would instill confidence that the effects of mutation on overall reduction could be credited to the PLR in question. Purifying to that point was, however, difficult due to the duration of purification. That is, once the proper parameters for culturing were in place (i.e. induction, culture time, and volume), it was only a matter of carefully growing enough bacteria to obtain the needed amount of protein. The purification process, however, was lengthy even after familiarization with the equipment and necessary

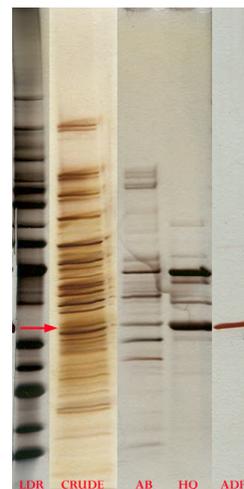


Figure 17:
Purification progression and the resulting band of native PLR_Fi1 (LDR = protein ladder; AB = AffiBlue affinity column)

steps, and the whole refinement scheme from harvest to ADP purification took well over a week each time. Working with a protein *in vitro* also posed problems of instability and subsequent loss of activity. From the moment the cells were lysed, the protein solution had to be kept at 4° C with fresh dithiothreitol. Degradation still occurred requiring that all work be done in a most timely manner. The stability problem was exacerbated by exposing the recombinant PLR_Fi1 to the various purification steps, and the extent of experimental manipulations adversely affected overall activity. The two chromatograms below illustrate the disparities that can exist from one protein batch to the next (Figure 18).

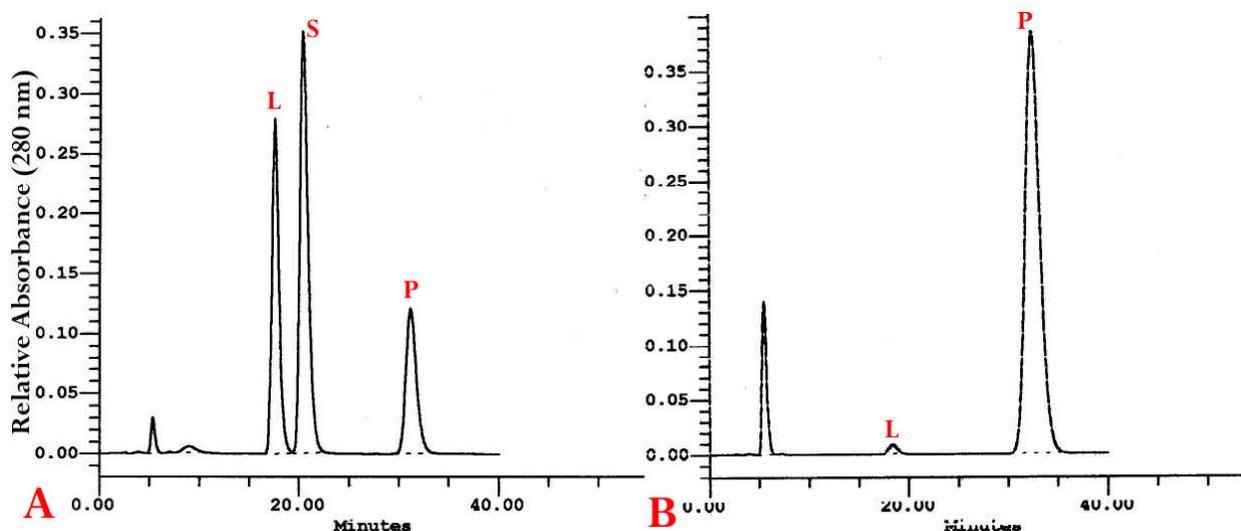


Figure 18: (A) 5 µg assay of HQ purified native; (B) separate occasion 31-µg assay of HQ purified native

PLR_Fi1 Mutation: Gly268Val

Having experimented with expression of the native gene and identifying challenges posed by working with it, a mutated version of the gene was next studied. This mutant became the experimental protein available for answering questions about the significance of a single amino acid in relation to the enantiospecificity of PLR_Fi1. Fortunately, the mutagenesis process worked quite well. The designed primers successfully annealed to the gene upon replication, and two DNA bases were forcibly changed as desired. The base triplet GGA coded for the original glycine at position 268. The primers were 40 base pairs long and matched the GGA segment identically except for two bases: TG. The guanine was conserved in the mutation, so the final result was the triplet GTG, coding for valine. With 19 base pairs flanking this two-base change, the annealing power overcame the mismatch and DNA polymerase replicated the gene with the mutation (Figure 19).

enantiomer. That is, the overall catalytic activity present in the crude lysate was at least an order of magnitude lower in the mutant. Furthermore, there was no indication of any change in enantiomeric preference.

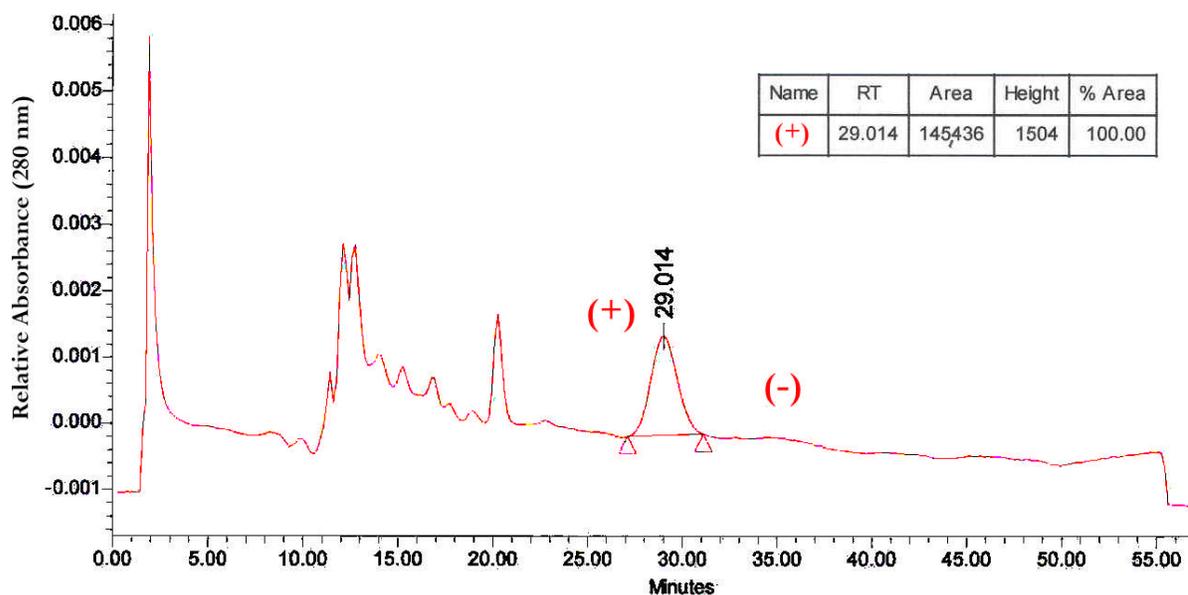


Figure 21: Chiral HPLC lariciresinol from assay with G268V_PLR_Fi1

Chiral HPLC analysis of the remaining pinoresinol present following the assay was accordingly unused because of the lower overall activity. The percent areas indicated that the pinoresinol sample was approximately racemic when compared to the standard (Figure 22).

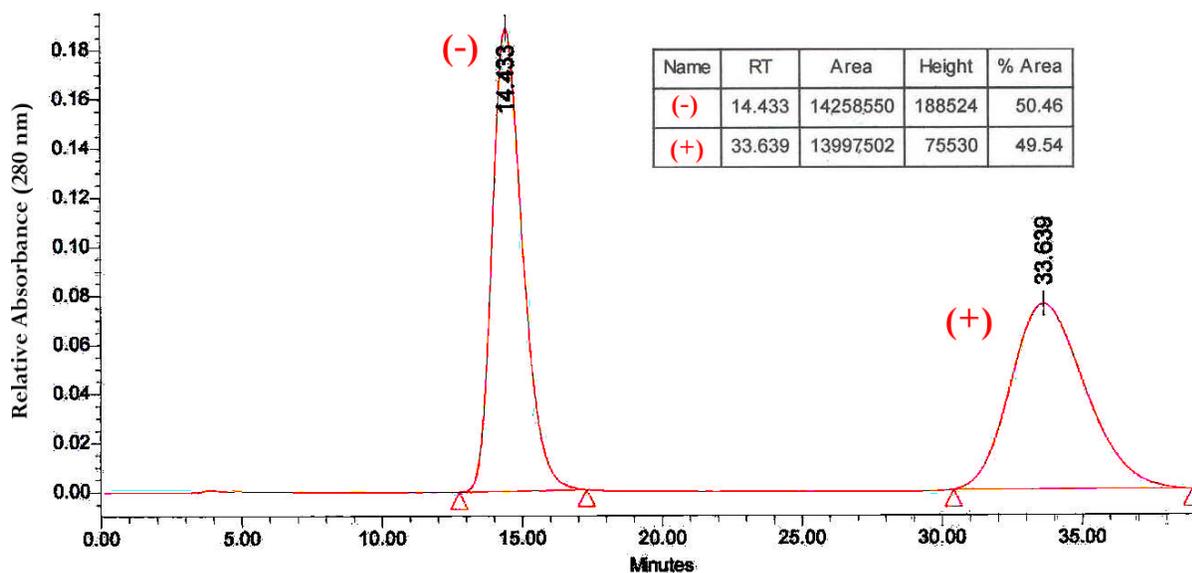


Figure 22: Chiral HPLC pinoresinol from assay with G268V_PLR_Fi1

Purification of Mutated G268V_PLR_Fi1

As described previously, crude samples contain additional proteins manufactured by the host cell that can interfere with enzyme assays. Although purity was achieved for the mutant G268V_PLR_Fi1 protein following the same protocol and procedures as with the native

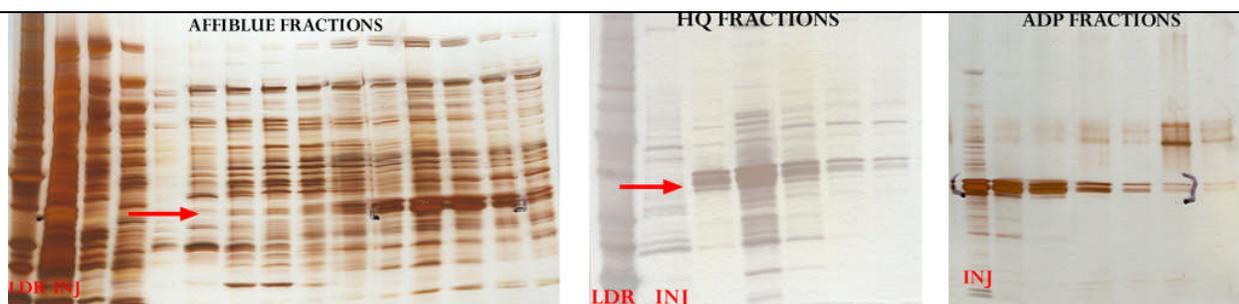


Figure 23: Purification progression of mutant protein, illustrating the successful isolation of G268V_PLR_Fi1

PLR_Fi1, as indicated by gels from the respective purification steps (Figure 23), no substantial activity could be achieved. As shown previously in the crude enzyme assays of the G268V_PLR_Fi1 mutant (Figure 20), overall catalytic activity was apparently reduced to a level barely allowing for collection and analysis of products. Despite even higher concentrations of protein at a greater purification factor, purified G268V_PLR_Fi1 displayed even further attenuation of activity (Figure 24), inhibiting further chiral analysis.

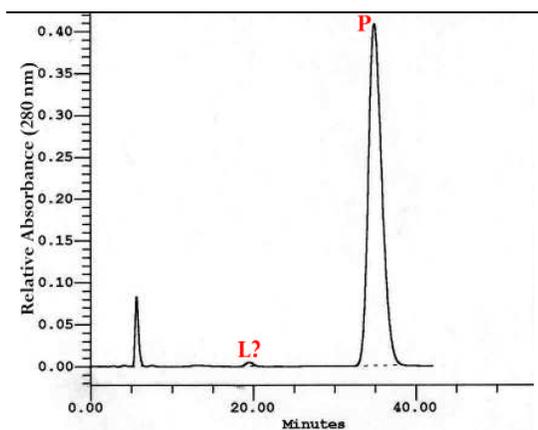


Figure 24: Post-HQ assay of G268V_PLR_Fi1 using maximum volume of protein (210 μ L unknown concentration)

SUMMARY

In retrospect, achieving such a high purification factor consumed time beyond the initial project's scope. Nevertheless, purification to this point provided protein (G268V_PLR_Fi1) that provisionally displayed much-reduced catalytic activity. This overall effect was manifested as a reduction in catalytic activity, and not enantiospecificity. Interestingly, a similar finding has been made by mutation of the corresponding residue of PLR_Tp2 (Hwang-Kim, 2006). In short, mutating position 268 in the PLR for *Forsythia intermedia* provisionally had a pronounced effect on the overall catalysis of substrate into product. This strongly suggests that Gly²⁶⁸ in PLR_Fi1 is indeed a functional component of the binding pocket for this reductase, but not for enantiospecificity. However, the biochemical/molecular basis for the reduction in catalytic activity remains to be determined.

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