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Partial Purification and Characterization of Isoflavone
Prenyltransferases from *Lupinus albus*.

Pierre Laflamme

A Thesis
in
The Department
of
Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada

October 1994

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ABSTRACT

Partial Purification and Characterization of Isoflavone
Prenyltransferases from *Lupinus albus*

Pierre Laflamme

Roots of *Lupinus albus* L. constitutively produce 6-, 8- and 3'- mono- and 6,3'-di-prenylated derivatives of the isoflavones genistein and 2'-hydroxygenistein. Similarly, cell suspension cultures produce the same complement of prenylated derivatives, however, 8-monoprenylated derivatives are synthesized to a much lesser extent.

Prenylation at the different positions of the isoflavone ring was shown to be catalyzed by distinct, position- and substrate-specific enzymes which were separated by anion-exchange chromatography. These enzymes were found to be integrally membrane bound and could be solubilized with detergents. The activity was quite labile once solubilized, particularly following column chromatography and efforts directed towards stabilizing the activity by the addition of glycerol, BSA, $MnCl_2$, and phospholipids proved unsuccessful.

Isoflavone prenyltransferase activity had a pH optimum between 6 and 6.5, was enhanced by the presence of Mn^{2+} , exhibited a preference for genistein, 2'-hydroxygenistein, and to a lesser extent, daidzein, and was inhibited by isopentenyl

2 pyrophosphate. Apparent K_m values for genistein and 2'-hydroxygenistein (prenyl acceptors) were 3.4 μM and 5.1 μM , respectively, while that for dimethylallyl pyrophosphate (prenyl donor) was 3.6 μM .

The *in vitro* prenylation of isowighteone (3'-prenylgenistein) yielding 3',5'-diprenylgenistein as the major product rather than the naturally occurring 6,3'-diprenyl, together with other findings, tends to suggest that isoflavone prenyltransferases are aligned in a specific order forming a linear multienzyme aggregate *in vivo*.

To Mom & Dad

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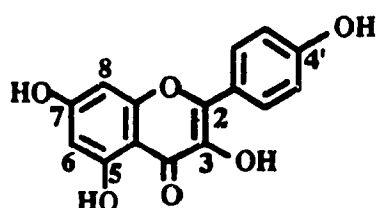
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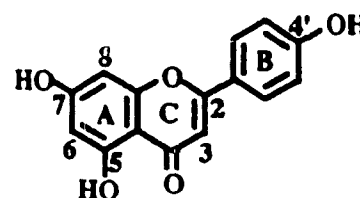
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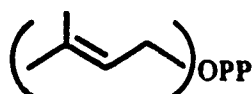
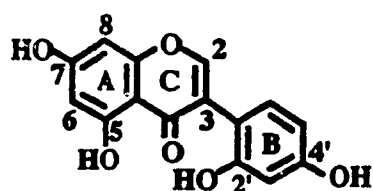
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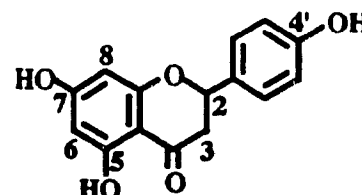
Flavonol



Flavone

Dimethylallyl pyrophosphate
(Prenyl)

Isoflavone



Flavanone

Compound Name	Generic Name
Biochanin A	5,7-Dihydroxy-4'- <u>O</u> -methylisoflavone
Daidzein	5-Deoxy-7,4'-dihydroxyisoflavone
2,3,-Dehydrokiefvitone	8-Prenyl-5,7,2',4'-tetrahydroxyisoflavone
Formononetin	5-Deoxy-4'- <u>O</u> -methyl-7,4'-dihydroxyisoflavone
Genistein	5,7,4'-Trihydroxyisoflavone
2'-Hydroxygenistein	5,7,2',4'-Tetrahydroxyisoflavone
2'-Hydroxylupalbigenin	6,3'-Diprenyl-5,7,2',4'-tetrahydroxyisoflavone
Isowighteone	3'-Prenyl-5,7,4'-trihydroxyisoflavone
Licoisoflavone A	3'-Prenyl-5,7,2',4'-tetrahydroxyisoflavone
Lupalbigenin	6,3'-Diprenyl-5,7,4'-trihydroxyisoflavone
Lupiwighteone	8-Prenyl-5,7,4'-trihydroxyisoflavone
Luteolin	5,7,3',4'-Tetrahydroxyflavone
Luteone	6-Prenyl-5,7,2',4'-tetrahydroxyisoflavone
Naringenin	5,7,4'-Trihydroxyflavanone
Quercetin	3,5,7,3',4'-Pentahydroxyflavonol
Wighteone	6-Prenyl-5,7,4'-trihydroxyisoflavone

LIST OF ABBREVIATIONS

Abbreviation	Name
2,4-D	2,4-dichloro-phenoxyacetic acid
β -ME	β -mercaptoethanol
bis-Tris	bis[2-Hydroxyethyl]imino-tris[hydroxymethyl] methane
BSA	bovine serum albumin
cv.	cultivar
Chaps	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate
DIECA	diethyldithiocarbamic acid, diethylammonium salt
DMAPP	dimethylallyl pyrophosphate
DOM	1-O-n-dodecyl- $[\alpha$ -D-glucopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside
IFPT(s)	isoflavone prenyltransferase(s)
IPP	isopentenyl pyrophosphate
kDa	kilodalton
L.	<i>Lupinus</i>
Mes	2-[N-Morpholino]ethanesulfonic acid
Nonidet P-40	ethylphenyl polyethylene glycol
PMSF	phenylmethanesulfonyl fluoride
Polyclar AT	poly [1-(2-oxo-1-pyrrolidinyl)ethylene]
PT(s)	prenyltransferase(s)
R.	<i>Rhizobium</i>
TLC	thin layer chromatography
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Triton X-100	4-(tert-octyl)-phenylpolyoxyethylene
SDS	sodium dodecyl sulfate
v/v	volume per volume
w/w	weight per weight

A. INTRODUCTION

Plants possess a superb ability to synthesize a variety of organic compounds, collectively known as secondary metabolites, of which flavonoids are known for their ubiquitous occurrence. Flavonoid compounds are believed to play an important role in the survival of plants. This includes protection against UV radiation [Schmelzer et al., 1988], as signal molecules in plant-microbe interactions [Long, 1989], and as antibiotics in plant defense responses [Dixon, 1986; Lamb et al., 1989].

Isoflavonoids constitute a distinct subclass of flavonoids and are characterized by a 2,3-aryl migration of ring B that is catalyzed by isoflavone synthase [Kochs & Grisebach, 1986] and by their limited distribution throughout the plant kingdom, being confined mainly within the Leguminosae family [Dewick, 1988]. It is interesting to note that prenylated isoflavonoids constitute a minor group when compared with the wide variety of other prenylated flavonoids which have been isolated to date [Tahara & Ibrahim, 1994].

Prenylated isoflavonoids and prenylated pterocarpans are among the commonly known phytoalexins. The latter are low molecular weight compounds which are synthesized as a result of stress imposed on the plant [Darvill & Albersheim, 1982; Smith & Banks, 1986], be it an organism feeding on a leaf, an infection due to an air- or soil-borne bacteria or fungi, an

osmic shock or perhaps mechanical wounding. In contrast with most legumes which accumulate prenylated isoflavonoids post-infectionally, the roots of white lupin possess the remarkable ability to constitutively express the biosynthesis of prenylated isoflavones both *in vivo* [Tahara et al., 1989] and *in vitro* [Hallard et al., 1992; Gagnon et al., 1992]. Consequently, these metabolites are being considered as pre-infectional compounds [Harborne et al., 1976]. Isoflavonoids are known to possess fungitoxic and antimicrobial properties [Tahara et al., 1984; Ingham et al., 1983; Harborne et al., 1976; Fukui et al., 1973], in addition to their role as deterrents to feeding insects [Lane et al., 1985]. Isoflavonoids are also involved in plant interactions with soil microbes [Phillips, 1992]. While some isoflavonoids may be estrogenically active [Shutt, 1976], certain others have recently been reported to reduce the rate of development of certain cancers [UC Berkeley Wellness Letter; Monsavi & Adlercreutz, 1993].

The prenylation of isoflavones in white lupin roots and cell suspension cultures is a constitutive event which renders these compounds more lipophilic, as well as enhancing their fungitoxicity [Tahara et al., 1984; Harborne & Ingham, 1978]. This is contrary to reports on isoflavonoid prenylation which have been observed after elicitation by either a yeast extract [Biggs et al., 1987] or a fungal cell-wall elicitor [Hamerski et al., 1990; Schröder et al., 1979; Zähringer et al., 1979;

Welle & Grisebach, 1991].

Lupinus albus (Fig. 1), therefore, provides an ideal system to study the enzymatic prenylation of isoflavones since it is constitutively expressed both *in vivo* and *in vitro*. The prenylated isoflavones which accumulate in white lupin are derived from both genistein (5,7,4'-trihydroxy isoflavone) and 2'-hydroxygenistein, with prenyl groups at positions 6-, 8- and 3'- [Tahara *et al.*, 1989].

Recent work has shown that different substitution patterns of various classes of flavonoids are the result of distinct, substrate- and position-specific enzymes. This has been demonstrated with the stepwise *O*-methylation of flavonols in *Chrysosplenium americanum* [De Luca & Ibrahim, 1985; Ibrahim *et al.*, 1987; Khouri *et al.*, 1988a; Khouri *et al.*, 1988b], *O*-glycosylation of flavonoids [Heller & Forkmann, 1988] and, in particular, of the partially *O*-methylated flavonols in *C. americanum* [Latchinian *et al.*, 1987], as well as the stepwise sulfation of flavonols in *Flaveria chloraefolia* and *F. bidentis* [Varin, 1992]. The fact that lupin isoflavones are monoprenylated at positions 6, 8 or 3' and diprenylated at 6,3' suggests that prenylation of isoflavones may also be catalyzed by a number of distinct, position-specific enzymes.

The scope of this study, therefore, is to demonstrate that the enzymatic prenylation of isoflavones in white lupin is catalyzed by distinct, position-specific enzymes, and attempt to purify the IFPT activity.

Figure 1. Photograph of a *Lupinus albus* plant. [courtesy of H.Gagnon].



B. AIM OF WORK

The aim of this project was: (a) to develop a reliable assay for isoflavone prenyltransferases (IFPTs) in order to (b) establish that the prenylation of isoflavones in *Lupinus albus* root tissue and cell suspension cultures is enzymatically catalyzed, (c) attempt to partially purify one or more of the PTs and establish their position specificity and (d) to investigate the PT activity in relation to root development and/or cell culture growth.

This study will contribute to our knowledge of the enzymatic prenylation of lupin isoflavones with respect to the order of prenylation vis-à-vis diprenylation. The outcome of this study will enable one to purify one or several isoflavone prenyltransferases (IFPT) and clone its gene. The ultimate goal would be to transform an agriculturally important crop plant such as *Glycine max* (soybean) in order to constitutively express prenyltransferase activity, thus improving its resistance to fungal infection. This can potentially reduce the need for the use of fungicides/pesticides.

C. LITERATURE REVIEW

C.1. Enzymatic Synthesis of Isoflavones

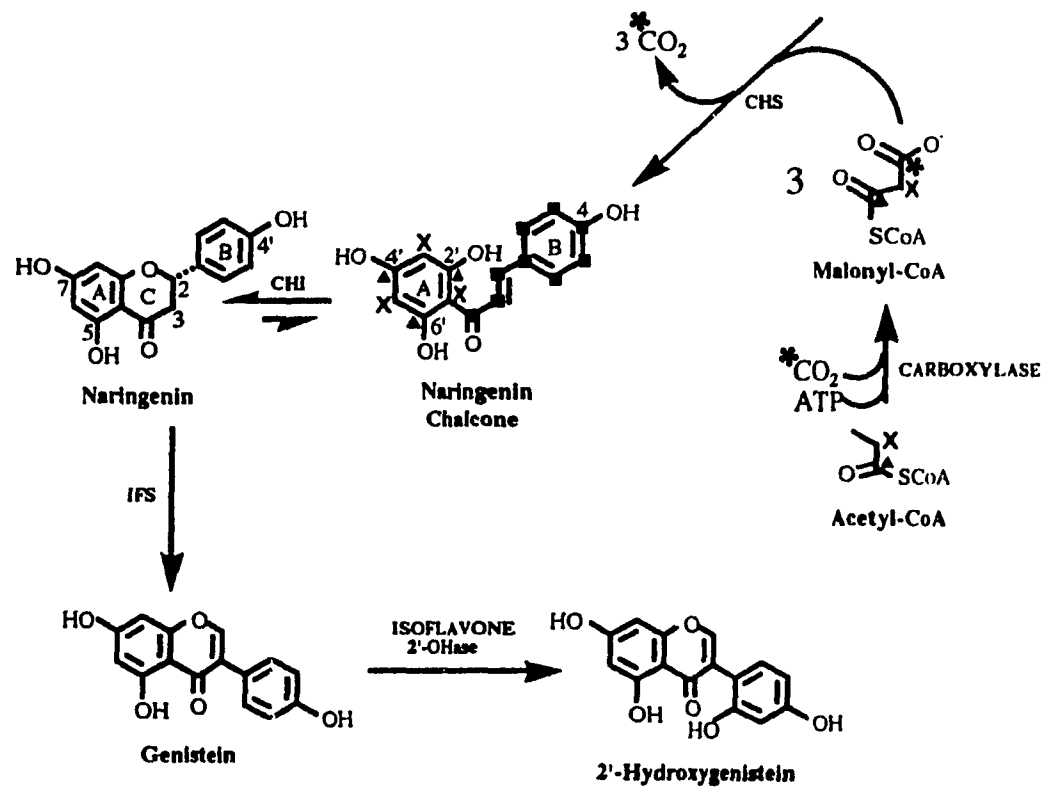
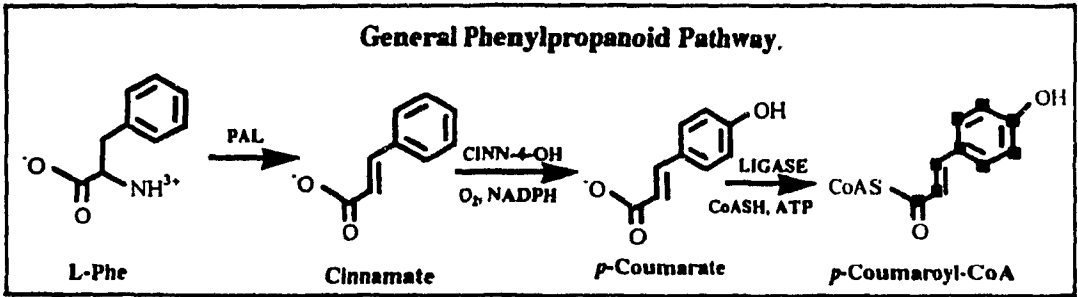
Isoflavones constitute a subgroup of the flavonoid-type of secondary metabolites. The distinguishing features of isoflavonoids are the 2,3-aryl migration of the phenyl group (ring B) on ring C and their limited distribution within the plant kingdom, being found mainly within the subfamily Papilionoideae of the Leguminosae [Dewick, 1988]. Like the flavonoids, isoflavone biosynthesis begins with the general phenylpropanoid pathway where phenylalanine is enzymatically deaminated to *trans*-cinnamic acid by L-phenylalanine ammonia lyase (PAL, EC 4.3.1.5). This is followed by hydroxylation of the latter product at position 4 by cinnamate 4-hydroxylase (C4H), and its transformation to the CoA ester by 4-coumarate:CoA ligase. 4-Coumarate-CoA forms ring B and the 3-carbon moiety of ring C, whereas ring A is formed from the stepwise condensation of 3 molecules of malonyl-CoA (Fig. 2) [Heller and Forkmann, 1988]. Flavanones, resulting from the action of chalcone isomerase, form the branch point in the pathway leading to the synthesis of isoflavones and later to the synthesis of other flavonoids.

C.1.1. Chalcone Synthase

Chalcone synthase (CHS, EC 2.3.1.74), a dimer with an apparent molecular weight of about 86 kDa catalyzes the

Figure 2. Enzymatic synthesis of isoflavones.

Enzymes: PAL, phenylalanine ammonia lyase
CINN-4-OH, cinnamate-4-hydroxylase
LIGASE, *p*-coumarate:CoA ligase
CARBOXYLASE, acetyl-CoA carboxylase
CHS, chalcone synthase
CHI, chalcone isomerase
IFS, isoflavone synthase
ISOFLAVONE 2'-OHase, isoflavone 2'-hydroxylase



stepwise condensation of 3 molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA to yield naringenin chalcone (2',4,4',6'-tetrahydroxychalcone) [Kreulazer & Hahlbrock, 1975]. CHS is considered to be a cytosolic enzyme [Beerhues & Wiermann, 1988] however, some sites of the enzyme may be associated with the cytoplasmic side of the endoplasmic reticulum [Hrazdina et al., 1987].

C.1.2. Chalcone Isomerase

Chalcone isomerase (CHI, EC 5.5.1.6), a monomeric enzyme with a molecular weight of 24-30 kDa, depending on its source [Dixon et al., 1988], catalyzes the isomerization of chalcones to their corresponding 2S-flavanones (Fig. 2). Two types of CHI are known, one acts on 5'-hydroxychalcones with phloroglucinol-type A-ring only, as in parsley [Kreuzaler & Hahlbrock, 1975] and tulip anthers [Chmiel et al., 1983], and the other cyclizes both types of A-ring substitution (6'-hydroxy- and 6'-deoxychalcones) as in bean cell culture [Dixon et al., 1982].

C.1.3. Isoflavone Synthase

Isoflavone synthase (IFS), a cytochrome P-450 monooxygenase requiring NADPH and O₂, catalyzes the conversion of (2S)-naringenin (a flavanone) to the corresponding isoflavone, genistein [Kochs & Grisebach, 1986]. This enzyme activity was demonstrated in elicitor-treated soybean cell

suspension cultures [Hagmann & Grisebach, 1984]. A hypothetical reaction sequence for the formation of an isoflavone from a flavanone, consistent with the participation of NADPH and molecular oxygen, was proposed [Hagmann & Grisebach, 1984]. IFS activity was found to be specific for the 2*S* enantiomers of naringenin and 5,4'-dihydroxyflavanone as substrates, but not their 2*R*-enantiomers [Kochs & Grisebach, 1986].

C.1.4. Isoflavone 2'-hydroxylase

Microsomal preparations from elicitor-treated soybean cell cultures were shown to catalyze the 2'-hydroxylation of genistein [Kochs & Grisebach, 1986]. Isoflavone 2'-hydroxylase activity was also observed in the microsomal fractions prepared from elicited cell suspension cultures of *Cicer arietinum* [Hinderer et al., 1987]. This activity catalyzed the hydroxylation of biochanin A (4'-OMe-genistein) and formononetin (5-deoxy-4'-OMe-isoflavone) at positions 2' and 3', respectively. 2'-Hydroxyisoflavones are known to serve as intermediates in the biosynthesis of pterocarpanes [Kochs & Grisebach, 1986].

C.2. Prenylation of Isoflavonoids

Isoflavonoids, like many other flavonoids, can undergo further ring substitution, such as hydroxylation, glycosylation, malonylation, methylation or prenylation. Since

little is known of isoflavonoid prenylation, the work presented in this thesis will focus on the prenylation of isoflavones in *Lupinus albus* L. [for a review of the different types of flavonoid ring substitution patterns, see Ibrahim & Varin, 1993].

C.2.1. The Prenyl Group Donor

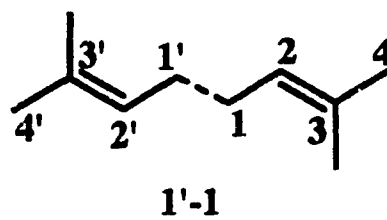
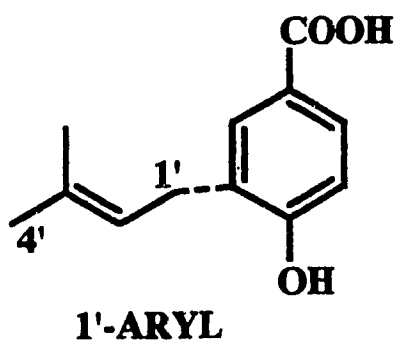
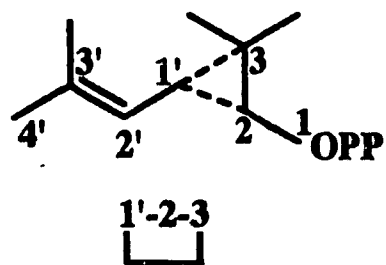
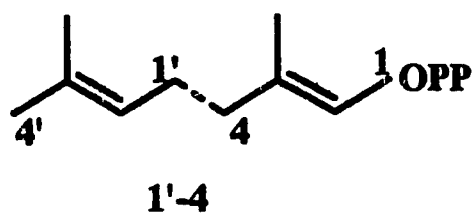
In the prenylation reaction, the acid-labile allylic pyrophosphate, dimethylallyl pyrophosphate (DMAPP), serves as the prenyl group donor. DMAPP is the reaction product of the enzymatic conversion of isopentenyl pyrophosphate (IPP) by isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase (IPP isomerase, EC 5.3.3.2). IPP isomerase has been partially purified from yeast, pig liver and various plant sources [Poulter & Rilling, 1981 and references cited therein]. More recently, Anderson and his group purified IPP isomerase to almost 3000-fold from yeast, thereby enabling the isolation of its gene [Anderson et al., 1989].

DMAPP can be synthesized chemically from dimethylallyl alcohol or dimethylallyl bromide. The synthesis involves a simple two-step procedure starting with isoprene, or a one-step from dimethylallyl bromide. The product, however, is very unstable and must, therefore, be stored under alkaline conditions, preferably at -20°C [Davisson et al., 1985].

C.2.2. The Prenyl Group Acceptor

The striking feature observed in the prenylation of isoflavonoids is that prenylation occurs *ortho* to a phenolic hydroxyl (1'-aryl type prenylation, Fig. 3). Examples of isoflavonoid prenyl acceptors are the pterocarpan, glyceollin from soybean that may be prenylated at positions 2 and/or 4 [Zähringer *et al.*, 1979; Welle & Grisebach, 1991], and phaseollin from bean at position 10 [Biggs *et al.*, 1987]. Coumarins can also act as prenyl acceptors to give rise to furanocoumarins. Particulate fractions prepared from leaves and cell suspension cultures of *Ruta graveolens* catalyzed the C-prenylation of umbelliferone at position 6 [Ellis & Brown, 1974; Dhillon & Brown, 1976]. In addition, Matern and his group have demonstrated the O-prenylation of umbelliferone at position 7, using an enzyme prepared from elicitor-treated *Ammi majus* cell suspension cultures [Hamerski *et al.*, 1990]. On the other hand, a geranyl transferase activity present in the microsomal preparations of *L. erythrorhizon* cell culture extracts was shown to catalyze the prenylation of *p*-hydroxybenzoic acid leading to the biosynthesis of shikonin [Heide & Tabata, 1987]. The prenylation at position 6 of the isoflavones genistein and 2'-hydroxygenistein has been demonstrated using a microsomal preparation from elicited *Lupinus albus* hypocotyls as a source for prenyltransferase activity [Schröder *et al.*, 1979]. It is important to note that all of the previously-mentioned findings were reported from

Figure 3. Various forms of prenylation reactions [Poulter & Rilling, 1981].



plant systems that were challenged either by an elicitor or by wounding.

C. 3. Isoflavonoids of *Lupinus albus* L.

Prenylated isoflavonoids have been detected in various species of the Leguminosae such as the prenylated pterocarpan of *Glycine max* [Zähringer et al., 1979; Welle & Grisebach, 1991], and *Phaseolus vulgaris* [Biggs et al., 1987], as well as the prenylated isoflavones wighteone and luteone in *Lupinus albus* [Schröder et al., 1979]. A number of prenylated isoflavonoids, including isoflavones, have been isolated from the roots of *Lupinus albus*. These consist of various mono- and di-prenylated derivatives of genistein and its 2'-hydroxy derivative [Ingham et al., 1983; Tahara et al., 1984; Tahara et al., 1989](Table 1). In addition, cell suspension cultures of *Lupinus albus* roots have the remarkable ability to synthesize the entire complement of isoflavonoids present in the parent tissue, and release them into the culture medium [Hallard et al., 1992; Gagnon et al., 1992]. In addition, it has recently been reported that *Lupinus luteus* (yellow lupin) accumulates the 8,3'-diprenyl derivative of genistein, isolupalbigenin [Tahara et al., 1994].

C.4. Prenyltransferases

The most commonly known prenyltransferases (PTs)(EC 2.5.1.1) are those involved in the 1'-4 linear condensation of

Table 1. Pre-infectional, fungitoxic compounds from *L. albus*.

Compound	Structure
Genistein	
2'-Hydroxygenistein	
Wighteone	
Luteone	
Isowighteone	
Licoisoflavone A	
Lupiwighteone	
2,3-Dehydrokievitone	
Lupalbigenin	
2'-Hydroxylupalbigenin	

isopentenyl pyrophosphate with an allylic pyrophosphate, and lead to the chain elongation reactions in polyisoprenoid biosynthesis (Fig. 3). This in turn serves as a key step in the formation of sterols, carotenoids, dolichols, isoprenoid quinones, and other various natural products [Poulter & Rilling, 1981]. The prenyl donor in this 1'-4 condensation is the allylic pyrophosphate, DMAPP, whereas isopentenyl pyrophosphate (IPP) serves as the prenyl acceptor. The reaction mechanism, enzymology and regulation of the prenyltransferase pathways have previously been reviewed [Poulter & Rilling, 1981].

C.4.1. Terpenoid Prenyltransferases

PTs involved in producing allylic pyrophosphates of 10, 15, 20 or more carbon atoms mediate the condensation of IPP with an allylic pyrophosphate to generate the next higher 5-C homologue of the allylic substrate. These serve as precursors to the various major groups of terpenoid compounds. Most of the PTs characterized to date apparently catalyze a multi-step, sequential reaction beginning with DMAPP and IPP as substrates, and terminating with a specific end product [Gershenzon & Croteau, 1990]. Such enzymes have been isolated from pumpkin fruit [Ogura *et al.*, 1972], castor bean seedlings [Dudley *et al.*, 1986], orange rind [de la Fuente *et al.*, 1981; Perez *et al.*, 1983], and more recently, from cell suspension cultures of grape [Clastre *et al.*, 1993]. Prenyltransferases

have also been purified from various animal and fungal sources [Poulter & Rilling, 1981, and references cited therein].

Plants are known to use a variety of mechanisms to control the levels of PT activity, suggesting that PTs may play an important role in regulating the biosynthesis of monoterpenes and other types of plant terpenoids [Gershenzon & Croteau, 1990].

One of the most extensively studied prenyltransferases is farnesylpyrophosphate (FPP) synthase which is involved in sterol biosynthesis. It has been purified to homogeneity from a number of sources, primarily *Saccharomyces cerevisiae* [Eberhardt & Rilling, 1975], and most recently from a recombinant, thermostable form of *Bacillus stearothermophilus* [Koyama et al., 1993].

C.4.2. Flavonoid Prenyltransferases

Whereas prenylated (prenyl, geranyl, farnesyl) flavonoids (chalcones, flavones, flavonols and their dihydro derivatives) are now known to be widely distributed in plants, there has been a remarkable lack of reports on their enzymatic prenylation.

C.4.2.1. Coumarin prenyltransferases

The earliest work on the prenylation of phenolic compounds dealt with furanocoumarin biosynthesis in *Ruta graveolens*. The dimethylallyl pyrophosphate:umbelliferone

dimethylallyl transferase was reported to catalyze the prenylation of umbelliferone at position 6 to yield demethyl-suberosin [Ellis & Brown, 1974]. This enzyme was shown to be membrane associated and required Mn^{2+} for maximal activity. It was partially purified and localized within the stroma and lamellar membranes of the chloroplast [Dhillon & Brown, 1976]. More recently, it has been demonstrated that elicitation of *Ammi majus* cell suspension cultures triggered two prenyltransferase activities, one was specific for position C-6 and the other for the 7-O position of umbelliferone. The 6-prenylation appeared to require Mg^{2+} , while the 7-O-prenylation could be carried out in the presence of either Mn^{2+} or Mg^{2+} [Hamerski et al., 1990]. Both activities were shown to be associated with the endoplasmic reticulum membrane.

C.4.2.2. Pterocarpan Prenyltransferases

Grisebach and his group studied the enzymatic prenylation of pterocarpan. By using a particulate fraction prepared from fungal elicitor-treated soybean cotyledons and cell suspension cultures, as a source for prenyltransferase activity, they demonstrated the prenylation of 3,6a,9-trihydroxypterocarpan (THP) at positions 2 and 4 [Zähringer et al., 1979; Zähringer et al., 1981]. Later work focused on the solubilization and attempted purification of these prenyltransferases [Welle & Grisebach, 1991]. A microsomal preparation from elicitor-treated cell suspension cultures of *Phaseolus vulgaris* was

shown to prenylate position 10 of 3,9-dihydroxypterocarpan (DHP) yielding phaseollidin [Biggs et al., 1987]. However, it was not evident whether prenylation at different positions of pterocarpan was catalyzed by one or several position-specific enzymes.

C.4.2.3. Isoflavone Prenyltransferases

Earlier work demonstrated the prenylation of genistein and its 2'-hydroxy derivative, at position 6, by a 45,000g particulate preparation from wounded or elicitor-treated *Lupinus albus* hypocotyls. This was shown to give rise to the 6-prenyl derivatives, wighteone and luteone, respectively [Schröder et al., 1979].

C.4.3. Purification of Prenyltransferases

The various methods used in the purification of PTs and cyclases have recently been reviewed [Alonso & Croteau, 1993 and references cited therein]. Some prenyltransferases from various plant sources have been purified to homogeneity [Dogbo & Camara, 1987; Clastre et al., 1993] while others have been partially purified [Green & West, 1974; de la Fuente et al., 1981; Croteau & Purkett, 1989; Heide & Berger, 1989; Suga & Endo, 1991; Hanley & Chappell, 1992]. Gel filtration was used to separate a farnesyltransferase activity from geranyltransferases I and II present in germinating castor bean. Separation of these two activities allowed for the

localization of the farnesyltransferase within the proplastid [Green et al., 1975]. *Citrus sinensis* was found to contain prenyltransferase activity catalyzing the condensation of IPP with DMAPP or GPP to form neryl- (*cis*- analogue of GPP) and geranyl pyrophosphate (GPP, C₁₀) as well as (2E,6E)- and (2Z,6E)-farnesyl pyrophosphate (FPP, C₁₅). Partial purification of the latter enzyme using gel filtration and ion exchange chromatography allowed for the resolution of the E- and Z-synthetase activities [de la Fuente et al., 1981]. Affinity chromatography using aminophenethyl pyrophosphate (APP), an analog of IPP, was used to purify to homogeneity a geranylgeranyl pyrophosphate (GGPP) synthase from the chromoplast stroma of *Capsicum annum* [Dogbo & Camara, 1987].

It had long been thought that a specific GPP synthase activity did not exist, and that any GPP formed, as an intermediate, in an enzymatic reaction, was catalyzed by FPP synthase. However, Croteau and Purkett [1989] localized a geranyl pyrophosphate synthase activity in leaf epidermal glands of *Salvia officinalis*. This cytosolic enzyme was partially purified by successive chromatography on Phenyl-Sepharose followed by gel filtration on Sephadex G-150. Furthermore, their localization studies suggested that this enzyme may be involved in monoterpene biosynthesis [Croteau & Purkett, 1989]. Using a similar purification scheme, Heide and Berger [1989] achieved partial purification of a geranyl pyrophosphate synthase from cell cultures of *Lithospermum*

erythrorhizon. It was purified 92-fold and was shown to have a mol. wt. of 73 kDa with an absolute requirement for either Mg^{2+} or Mn^{2+} , as a divalent cation. Its role is believed to supply GPP, as an intermediate, in the biosynthesis of the naphthoquinone pigment shikonin [Heide & Berger, 1989]. Recently, a Japanese group separated a GPP synthase activity from FPP synthase activity in *Pelargonium roseus* leaves by hydrophobic interaction chromatography on Butyl-Toyopearl 650. This GPP synthase was localized in a membrane fraction and was activated by Mn^{2+} rather than Mg^{2+} [Suga & Endo, 1991]. The same group used this hydrophobic support to separate GPP synthase from FPP synthase activities found in *Mentha spicata*, *Cinnamomun camphora*, *C. camphora* Sieb. var. *linalooliferum*, *Euphorbia pulcherrima* and *Morus bombycis* [Endo & Suga, 1992]. More recently, a geranyl diphosphate synthase was purified to homogeneity from cell suspension cultures of *Vitis vinifera* L. cv. Muscat de Frontignan [Clastre et al., 1993].

Squalene synthase which catalyzes the condensation of two FPP molecules to form squalene, an intermediate in sterol biosynthesis, was partially purified from cell suspension cultures of *Nicotiana tabacum* [Hanley & Chappell, 1992]. Purification of squalene synthase was achieved by a two-step solubilization procedure, followed by a combination of ultrafiltration, gel-filtration and anion exchange chromatography. The partially purified enzyme was then used to produce polyclonal antibodies which, in turn, allowed for

investigating the mechanism controlling the suppression of squalene synthase activity [Hanley & Chappell, 1992]. Poulter's group recently cloned a truncated form of the yeast squalene synthase gene, lacking the hydrophobic C-terminal, into *E. coli*. The soluble recombinant enzyme was purified to homogeneity by two chromatographic steps, and displayed similar catalytic properties to those of the wild-type microsomal enzyme [Zhang *et al.*, 1993].

C.4.4. Protein prenyltransferases

Proteins have recently been shown to be prenylated as a result of post-translational modification. Protein prenylation involves the transfer of either a farnesyl (C_{15}) or a geranylgeranyl (C_{20}) group [Marshall, 1993]. Prenylated proteins are involved in the proliferation of cancer cells, protein cycling between the cytosol and membranes [Marshall, 1993], and the regulation of trans-membrane signalling systems [Inglese *et al.*, 1992]. More recently, however, a protein farnesyltransferase cDNA was prepared from *Pisum sativum* [Yang *et al.*, 1993].

C.5. Significance of Isoflavones in Nature

C.5.1. Phytoalexins

Phytoalexins are low molecular weight compounds which are synthesized *de novo* following microbial infection or challenge with biotic or abiotic elicitors [Darvill & Albersheim, 1984].

Isoflavones are a minor group among the various flavonoid phytoalexins known to date. The majority of isoflavonoid phytoalexins consist of pterocarpan [for reviews on phytoalexins and isoflavonoid phytoalexins in particular, see Dixon *et al.*, 1983; Ingham, 1983; Kuc & Rush, 1985; Smith & Banks, 1986]. Most of the known isoflavonoid phytoalexins are produced in the Leguminosae family (Table 2). In fact, pisatin from *Pisum sativum* was the first isoflavonoid phytoalexin to be discovered [Cruikshank & Perrin, 1960]. The glyceollins of soybean are prenylated trihydroxypterocarpan which possess phytoalexin activity [Klarman & Sanford, 1968; Burden & Bailey, 1975; Lyne *et al.*, 1976; Lyne & Mulheirn, 1978]. Similarly, the prenylated dihydroxypterocarpan from bean, phaseollidin, is also a phytoalexin [Cruikshank *et al.*, 1974]. Prenylation of isoflavonoids is known to increase their lipophilicity, thereby enabling them to interact or even cross cellular membranes. It is believed that one of the ways by which isoflavonoid phytoalexins exert their inhibitory effect on invading pathogens is by interacting with the pathogen's cellular membrane system and altering its structure, thus causing the leakage of their cellular content [Smith & Banks, 1986]. Therefore, it can be suggested that prenylation of isoflavonoids serves to enhance their phytoalexin activity.

Wighteone, a prenylated isoflavone produced in fungus-inoculated stems of *Glycine wightii* was shown to have phytoalexin-type activity that is more fungitoxic than the

Table 2. Some isoflavonoid phytoalexins of the Leguminosae.

Phytoalexin	Plant*	Reference
Pisatin	<i>P. sativum</i>	Cruikshank & Perrin, 1960.
Phaseollin	<i>Ph. vulgaris</i>	Biggs et al., 1987. Cruikshank & Perrin, 1971.
Wighteone	<i>G. wightii</i>	Ingham et al., 1977.
3,6a,9-Trihydroxypterocarpan	<i>G. max</i>	Zähringer et al., 1979. Lyne & Mulheirn, 1978. Weinstein et al., 1981.
Genistein	<i>Ph. vulgaris</i>	Biggs, 1975.
2'-Hydroxygenistein	<i>Ph. vulgaris</i> <i>C. cajan</i>	Biggs, 1975 Ingham, 1976.
Kievitone	<i>Ph. vulgaris</i> <i>V. sinensis</i>	Smith et al., 1973. Partridge & Keen, 1976. Burden et al., 1972.
Phaseollidin	<i>Ph. vulgaris</i>	Cruikshank et al., 1974.
Glyceollin I	<i>G. max</i>	Klarman & Sanford, 1968. Burden & Bailey, 1975.
Glyceollin I II III IV	<i>G. max</i>	Lyne et al., 1976. Lyne & Mulheirn, 1978.

*C., *Cajanus*; G., *Glycine*; P., *Pisum*; Ph., *Phaseolus*; V., *Vigna*

parent aglycone, genistein. This was thought to be due to its increased lipid solubility as a result of the prenyl group [Ingham et al., 1977].

There still remains some unanswered questions as to the biochemical events that take place from the time of infection by a pathogen to the time phytoalexins are produced. Nevertheless, Ebel and Grisebach [1988] have postulated that, in soybean, the defensive response to infection by a fungal pathogen may involve a signal transduction mechanism.

Most of the isoflavonoids which have been isolated from white lupin, including the prenylated isoflavones, appear to be constitutively produced. Therefore lupin isoflavonoids may be considered as pre-infectional agents possessing fungitoxic and, in some cases, anti-microbial activity.

C.5.2. Pre-infectional agents, insect feeding deterrents and estrogenically active compounds

The isoflavones genistein and 2'-hydroxygenistein, as well as their mono- and di-prenylated derivatives have been shown to have some fungitoxic activity (Table 1) [Tahara et al. 1984; Tahara et al. 1989]. Luteone, for instance, was shown to inhibit the germination of *Cochliobolus miyabeanus*. It also possesses antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Trichophyton mentagrophytes* and *T. rubrum* [Fukui et al., 1973]. Using a TLC plate bioassay developed by Homans and Fuchs [1970], wighteone

and luteone were shown to be more fungitoxic than their respective aglycones, genistein and 2'-hydroxygenistein, against *Cladosporium herbarum* [Ingham et al., 1983]. Compared with biochanin A and formononetin, luteone, a leaf surface constituent of *L. albus* was shown to inhibit the mycelial growth of *Helminthosporium carborum* [Harborne et al., 1976]. Licoisoflavone A was shown to be as fungitoxic as luteone and equal to, or slightly better than, wighteone against *Cladosporium herbarum*, followed by genistein, 2'-hydroxygenistein and their diprenylated derivatives [Tahara et al., 1984]. The isoflavones isopiscerythron (5,7,2',4'-tetrahydroxy-5'-methoxy-6-dimethylallyl isoflavone), allolicoisoflavone A (5,7,2',4'-tetrahydroxy-5'-dimethylallyl isoflavone), piscisoflavone A (7,2',4'-trihydroxy-5'-methoxy-3'-dimethylallyl isoflavone) and pisciisoflavone B (7,2'-dihydroxy-5'-methoxy-dimethylpyrano-isoflavone) isolated from the root bark of *Piscidia erythrina* (Jamaican dogwood) all showed antifungal activity towards the growth of *Cladosporium herbarum* [Miriyaama et al., 1992]. More recently, it has been shown that 2'-hydroxylupalbigenin inhibits the growth of the symbiotic bacterium *Rhizobium lupini* [Gagnon, 1993].

Besides being fungitoxic, 2'-hydroxygenistein and phaseollin (a pterocarpan) can act as insect feeding deterrents [Lane et al., 1985], while formononetin [Shutt, 1976] and genistein [Miksicek, 1993] possess estrogenic activity.

C.5.3. Isoflavonoids as enzyme inhibitors and antitumor agents

Phenolic compounds, including flavonoids are known to inhibit enzyme activity and isoflavonoids seem to be no exception. Genistein, capable of inhibiting tyrosine kinases [Akiyama et al., 1987] was recently shown to inhibit the proliferation of cancer cells [Fotsis et al., 1993; Peterson & Barnes, 1991]. Similarly, biochanin A can potentially be used as a therapeutic agent against cancer, as it was also shown to inhibit tumour growth [Yanagihara et al., 1993].

Genistein, 2'-hydroxygenistein and wighteone have recently been shown to inhibit the oxidation of indole-3-acetic acid (IAA) by isoperoxidases from lupin hypocotyls [Ferrer et al., 1992]. Thus suggesting that isoflavonoids may play a role in the regulation of growth hormones and subsequently plant growth.

C.5.4. Isoflavonoids and plant-microbe interactions

Leguminous plants have the remarkable ability to grow in soils which have a low nitrogen content. This is due to the symbiotic relationship which they develop with *Rhizobium* spp. and which results in the formation of root nodules that fix atmospheric nitrogen. Once these roots are infected, the bacteria inhabit the root system, thereby reducing molecular nitrogen into ammonia. The plant can then utilize the ammonia as a N-source to synthesize organic nitrogen compounds, such

as the amino acid glutamine. In return, the bacteria benefit from the plant's ability to reduce CO₂ into sugars which it uses as fuel [Long, 1989]. This association between plants and rhizobia may therefore be of significant agronomical importance [Fisher & Long, 1992]. Three genera of the gram-negative Rhizobiaceae are known to be associated with legumes: *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* [Hirsch, 1992]. Rhizobial genes are required for nodule formation and nodulation (*nod*) gene expression.

Flavonoids excreted from plant roots have been shown to be involved in regulating *nod* gene expression [Nap & Bisseling, 1990]. Some flavonoids may act as inducers of *nod* genes, while others have been shown to be inhibitors of that process. Whereas formononetin has been shown to inhibit *nod* gene expression [Djordjevic et al., 1987], genistein and/or daidzein are capable of both inhibiting [Firmin et al., 1986], as well as inducing [Kristnan & Pueppke, 1993; Kosslak et al., 1987; Sadowsky et al., 1988; Kosslak et al., 1987] the expression of *nod* genes. However, the precursor in the synthesis of medicarpin, formononetin-7-O-(6"-O-malonylglucoside) [Dakora et al., 1993] and the 7-O-glycoside of genistein [Hungria et al., 1991] both act as *nod* gene inducers. Recently, Cho and Harper [1991] speculated that nodulation may be regulated by the levels of isoflavonoids in plant roots.

In addition to stimulating growth and inducing nodulation

genes in *Rhizobium* spp., flavonoids also enhance the spore germination of certain mycorrhizal fungi [for a review, see Phillips, 1992].

D. MATERIALS AND METHODS

D.1. Plant Material

D.1.1. *Lupinus albus* seedlings and cell cultures

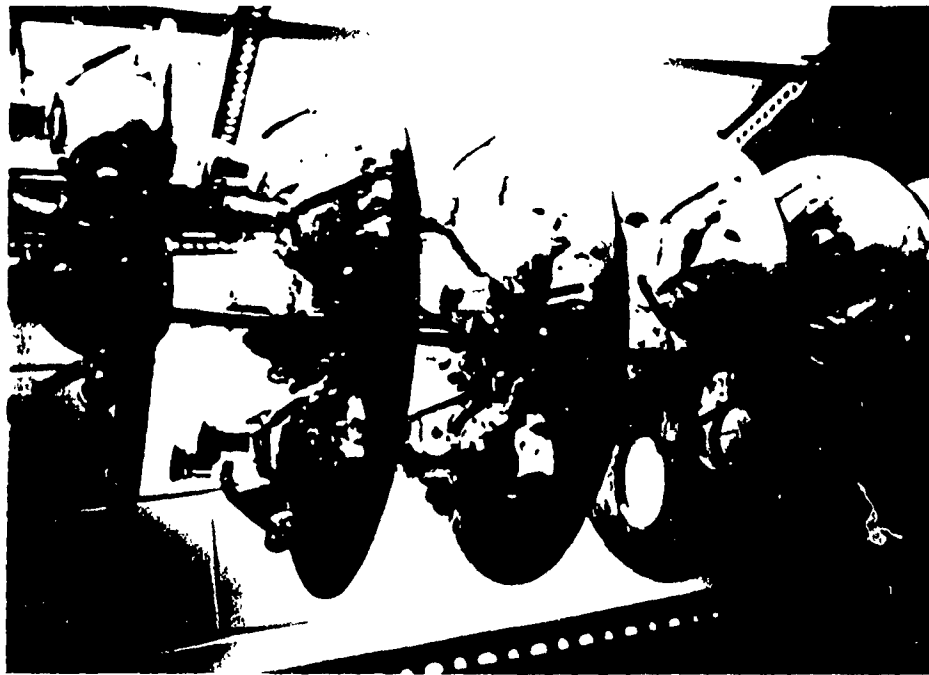
Seeds of *Lupinus albus* (cv. Kievskij) were kindly provided by Dr. S. Tahara, Hokkaido University, Sapporo, Japan and those of cv. Primovski were obtained from Labon Inc., Boucherville, Qué. They were germinated in vermiculite under greenhouse conditions.

Callus cultures from *L. albus* (cv. Kievskij) radicles and hypocotyls were initiated by Dr. E. Bleichert on B5-agar medium [Gamborg et al., 1968] containing 2% sucrose, 1 ppm 2,4-D, and 0.1 ppm kinetin. Suspension cultures were established by transferring the callus formed to rotating, one-liter nipple flasks [Steward et al., 1952] containing 250 ml liquid medium of the same composition (Fig. 4). Cultures were harvested and/or subcultured on a fourteen-day cycle.

D.2. Chemicals

[³H]-Dimethylallyl pyrophosphate (DMAPP) (sp. act. 10-15 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO.). ENHANCE was purchased from NEN Du Pont Canada Inc. (Mississauga, Ont.). Chaps {3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate} and DOM {1-O-n-dodecyl-[α -D-glucopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside} were obtained from Boehringer Mannheim (Laval, Qué.) and Nonidet P-40

Figure 4. Photograph of *L. albus* cell suspension culture set-up. Cultures were maintained in the light, at room temperature, in 1-L nipple spherical flasks. These flasks have ten symmetrically spaced cylindrical protrusions (approximately 2 cm long and wide) and rotate centripetally at 4 rpm.



(ethylphenyl polyethylene glycol) from Sigma (St. Louis, MO). Triton X-100 [4-(tert-octyl)-phenylpolyoxyethylene] was purchased from BDH (St. Laurent, Qué.). Tris (2-amino-2-hydroxymethyl-1,3-propanediol), $MnCl_2$, β -ME, NaF, and glutathione were of reagent grade. Ultra pure glycerol was purchased from ICN (Montréal, Qué.). Genistein and 2'-hydroxygenistein as well as their mono- and diprenylated derivatives were generous gifts from Dr. S. Tahara (Hokkaido University, Sapporo, Japan).

D.3. Buffers

The following buffers were used: (A) 100 mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol, 1mM phenylmethylsulfonyl fluoride (PMSF), and 14 mM β -ME; (B) 20 mM Tris-HCl, pH 7.5, containing 20% (v/v) glycerol, 14 mM β -ME, 5% (w/v) betaine, and 0.04% (w/v) DOM; (C) 100 mM Tris-HCl, pH 7.5, containing 12 mM $MnCl_2$, 30 mM NaF and 12 mM glutathione; (D) 20 mM Tris-HCl, pH 7.5, containing 10% (v/v) glycerol, 0.1% (w/v) DOM and 14 mM β -ME; (E) 20 mM Tris-HCl, pH 7.5, containing 10% (v/v) glycerol and 14 mM β -ME. All buffer solutions were degassed under vacuum prior to addition of the antioxidant.

D.3.1. Prenyltransferase extraction

Microsomal pellets were prepared from ten day-old radicles or fourteen day-old cell suspension cultures. All extraction steps were carried out at 4°C. Twenty-five to

thirty grams of cultured cells or radicles were homogenized with a mortar and pestle in buffer A (1:4 w/v), together with Polyclar AT (20% w/w) and washed sand (10% w/w). The homogenate was squeezed through one layer of miracloth, and the filtrate was mixed for 20 minutes with Dowex 1-X2 (10% w/v) which had previously been equilibrated in buffer A. The suspension was centrifuged at 8000g for 10 minutes, and $MgCl_2$ (from a 1 M stock solution) was slowly added to the supernatant to a final concentration of 30 mM. Following $MgCl_2$ precipitation, the particulate fraction was collected by centrifugation for 20 minutes at 200,000g using a Beckman 70.1 Ti rotor. The resulting pellet was washed by resuspending in a minimal amount of buffer A (typically 2.5 ml for a pellet prepared from 25 g of tissue) with a hand-held Potter homogenizer followed by centrifugation at 200,000g for a further 20 minutes. The washed microsomes were then resuspended in either buffer A or buffer B prior to column chromatography.

D.3.2. Solubilization of enzyme activity

The resuspended, washed microsomal pellets were treated with detergents in a ratio of 24:1 (v/v) to yield a final concentration of 0.75% (w/v) Chaps, 2% (w/v) DOM, 0.2% (v/v) Nonidet P-40, and 0.1% (v/v) Triton X-100 in buffer A. The suspensions were stirred gently on ice for 45 min, then centrifuged for 20 min at 200,000g using a Beckman 70.1 Ti

rotor. The resulting pellets were resuspended in buffer A, and both supernatants and pellets were assayed for prenyltransferase activity. The supernatants from DOM-treated pellets were then used for liquid chromatography

D.3.3 Chromatography of protein extract

D.3.3.1. Gel filtration chromatography on Sephacryl S-400

The supernatant from the DOM-solubilized microsomal pellet, containing 5 mg protein, was loaded onto a Sephacryl S-400 column (35 cm x 15 mm I.D.) (Pharmacia Canada Inc., Baie d'Urfé, Qué.) at a flow rate of 1.0 ml/min. The column was equilibrated and developed with buffer D. One-ml fractions were collected and assayed for enzyme activity.

D. 3.3.2. Ion exchange chromatography on Fractogel TMAE-EMD 650 (S)

The supernatant from the DOM-solubilized pellet (typically 5 to 10 mg protein) was applied onto a Fractogel TMAE-EMD 650 (S) column (3 cm x 10 mm I.D.) (E. Merck, Darmstadt) previously equilibrated with buffer B at a flow rate of 0.5 ml/min, using an FPLC system (Pharmacia). The column was washed with 10 ml of the same buffer and the bound proteins were eluted using 50 ml of a linear gradient (0-250 mM) of KCl in buffer B. Half-ml fractions were collected and assayed for prenyltransferase activity.

D.3.3.3. Ion exchange chromatography on Mono Q

Five to eight mg of DOM-solubilized protein was loaded onto a Mono Q HR 5/5 column (50 x 5 mm I.D.) (Pharmacia) at a flow rate of 0.5 ml/min, using an FPLC system (Pharmacia). The column was equilibrated with buffer B and washed with 10 ml of the same buffer following sample application. Elution was carried out using 50 ml of a linear gradient (0-250 mM) of KCl in buffer B. Fractions of 500 μ l were collected and assayed for prenyltransferase activity.

D. 3.4 Electrophoresis of protein extracts

D.3.4.1. Native polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out under non-denaturing conditions, at 4°C. Typically, 5 to 10 mg of DOM-solubilized protein was layered onto two, 1.5 mm thick 7.5% acrylamide slab gels with 4% stacking gels. The solutions used were of the same composition as those used for denaturing (SDS) PAGE, according to the method of Laemmli [1970], but without SDS and including 0.1% (w/v) DOM. The running buffer consisted of 25 mM Tris-192 mM glycine, pH 7.5 containing 14 mM β -ME and 0.1% (w/v) DOM. The gels were pre-run for 45 min prior to electrophoresis which was carried out at a constant voltage of 150V for approximately 1.5 to 2 hr. Following electrophoresis, a 1.5 cm vertical strip of gel was sliced off and stained with a 0.1% solution of Coomassie blue R-250. The remaining gels were sliced into 2 mm horizontal strips,

homogenized in an eppendorf tube with 500 μ l of buffer A and assayed for prenyltransferase activity.

D.3.4.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts following column chromatography were analyzed by gel electrophoresis under denaturing conditions according to the method of Laemmli [1970] using 7.5% acrylamide gels and the Mini-Protean II Gel Electrophoresis Apparatus (Bio-Rad Laboratories Ltd.). The proteins were visualized by staining with Coomassie blue.

D.3.5. Sucrose density gradients

All manipulations were carried out at 4°C. Approximately 10 to 15 g of 14 day-old lupin cell suspension cultures or 10 day-old radicles was finely chopped with a razor blade in 20-30 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 8.5% (w/v) sucrose, 0.2% (w/v) DIECA, 1 mM PMSF, 14 mM β -ME), followed by a 10-sec homogenization using a Polytron tissue homogenizer. The homogenate (25 ml) was filtered through two layers of nylon mesh and 5 ml was applied to each sucrose gradient.

Continuous sucrose gradients (25 ml) ranging in concentration from 10% to 70% (w/v) with 4 ml of a 77% sucrose cushion were initially used. The sucrose solutions were prepared in 50 mM Tris-HCl, pH 7.5 to which β -ME (final

concentration of 14 mM) was added prior to use.

The gradients were centrifuged at 105,000g for 3 hr using a Beckman SW-28 rotor. Fractions of 1.5 ml were collected from the bottom of the gradients by puncturing the tubes with a needle, and diluted with an equal volume of buffer (50 mM Tris-HCl, pH 7.5, 14 mM β -ME, 1mM PMSF) in order to decrease the sucrose concentration. The organelles in each of the fractions were collected by centrifugation at 48,000g for 20 min, using a Beckman type 30 rotor. The pellets were resuspended with 500 μ l of 100 mM Tris-HCl, pH 7.5, 20% (v/v) glycerol, 14 mM β -ME and every third fraction was assayed for lupin IFPT activity. The fractions with the highest activity were viewed under the light microscope for identification of organelles.

Discontinuous sucrose gradients consisting of 8 ml each of 30%, 50% and 70% sucrose (w/v) were used in subsequent experiments. The bands sedimenting above each layer were removed from the top using a pasteur pipette. These fractions were diluted with an equal volume of buffer E and centrifuged at 48,000g for 20 min with a Beckman type 30 rotor. The pellets were resuspended to 2 ml with buffer E and assayed for IFPT activity.

D. 4. Prenyltransferase Assay

The standard assay mixture consisted of 40 μ M of the isoflavone substrate dissolved in ethylene glycol monomethyl

ether (final concentration 1%), 33-50 nM of [³H]-DMAPP, containing 0.05 μCi, and buffer C in a total volume of 100 μl. The reaction was started by addition of the enzyme preparation and the mixture was incubated for 30 min at 30°C. The reaction was terminated by the addition of 250 μl of ethyl acetate. An aliquot of the organic phase was transferred to a scintillation vial and counted for radioactivity. The remaining organic layer was used for product identification using TLC.

D. 5. Identification of Reaction Products

The ethyl acetate extracts of several assays were pooled and evaporated to dryness. The dry residue was resuspended in a minimal amount of high grade methanol and chromatographed on commercial Kieselgel 60 F-254 TLC plates (Merck, Darmstadt) in *n*-pentane-diethyl ether-acetic acid (15:5:1) as the solvent system. Developed chromatograms were allowed to dry, sprayed with ENHANCE and autoradiographed on X-ray film. The identity of the prenylated products was verified by co-chromatography with authentic samples and fluorescence in UV light.

D.6. Determination of Product Ratios

The radioactive spots corresponding to each reaction product were scraped off the TLC plates, counted for radioactivity and their relative ratios determined.

D.7. Definition of Enzyme Activity Units

The enzyme units used throughout this study were expressed in katal (kat) as recommended by the International Union of Biochemistry (IUB, 1973). One kat is defined as the amount of enzyme activity required to convert one mol of substrate per second under the assay conditions.

D.8. Protein Determination

The amount of protein was determined according to the method of Bradford [Bradford, 1976] using the Bio-Rad protein reagent and bovine serum albumin as a standard protein.

D.9. Analysis of Kinetic Data

Lineweaver-Burk reciprocal plots of the data obtained were generated using the ENZFITTER program (by R.B Leatherbarrow, U.K., distributed by BIOSOFT, U.K.), in order to determine k_m values for the isoflavone substrates and the prenyl donor. The data was fitted to the linear form of the Henri-Michaelis-Menten equation:

$$1/V = (K_m/V_{max})(1/[S]) + 1/V_{max}$$

where [S] represents the concentration of the varied substrate, the cosubstrate being present in fixed amounts. The intercepts on the abscissa, when $(1/V) = 0$, is then equal to $-1/K_m$.

E. RESULTS

E.1. Characteristics of the Prenylation Reaction.

E.1.1. Reaction products

Isoflavone prenyltransferases (IFPTs) catalyze the transfer of the prenyl group from DMAPP to positions 6-, 8-, or 3'- of the isoflavones genistein (5,7,4'-trihydroxy isoflavone) and 2'-hydroxygenistein (Fig. 5). These enzymes appear to be membrane-associated, since the activity was found mainly in the microsomal pellets following centrifugation at 200,000g (Table 3). Whereas microsomal pellets prepared from radicles catalyzed the prenylation of both genistein and 2'-hydroxygenistein to their 6-, 8- and 3'- monoprenylated derivatives, those prepared from cell suspension cultures catalyzed the prenylation of both substrates to their 6- and 3'- monoprenyl derivatives only (Fig. 6). In addition, the latter preparations mediated the prenylation of isowighteone (3'-monoprenyl genistein) to the 3',5'-diprenyl derivative (Fig. 7).

E.1.2. Effect of pH

The pH optimum for isoflavone prenyltransferase activity, as determined in different buffers, was found to be between 6.0 and 6.5 (Fig. 8). Maximum prenyltransferase activity was observed in Mes-HCl, bis-Tris-HCl, and imidazole-HCl buffers.

Figure 5. Prenylation of positions 6-, 8- and 3'- of the isoflavones genistein and 2'-hydroxygenistein in *L. albus*.

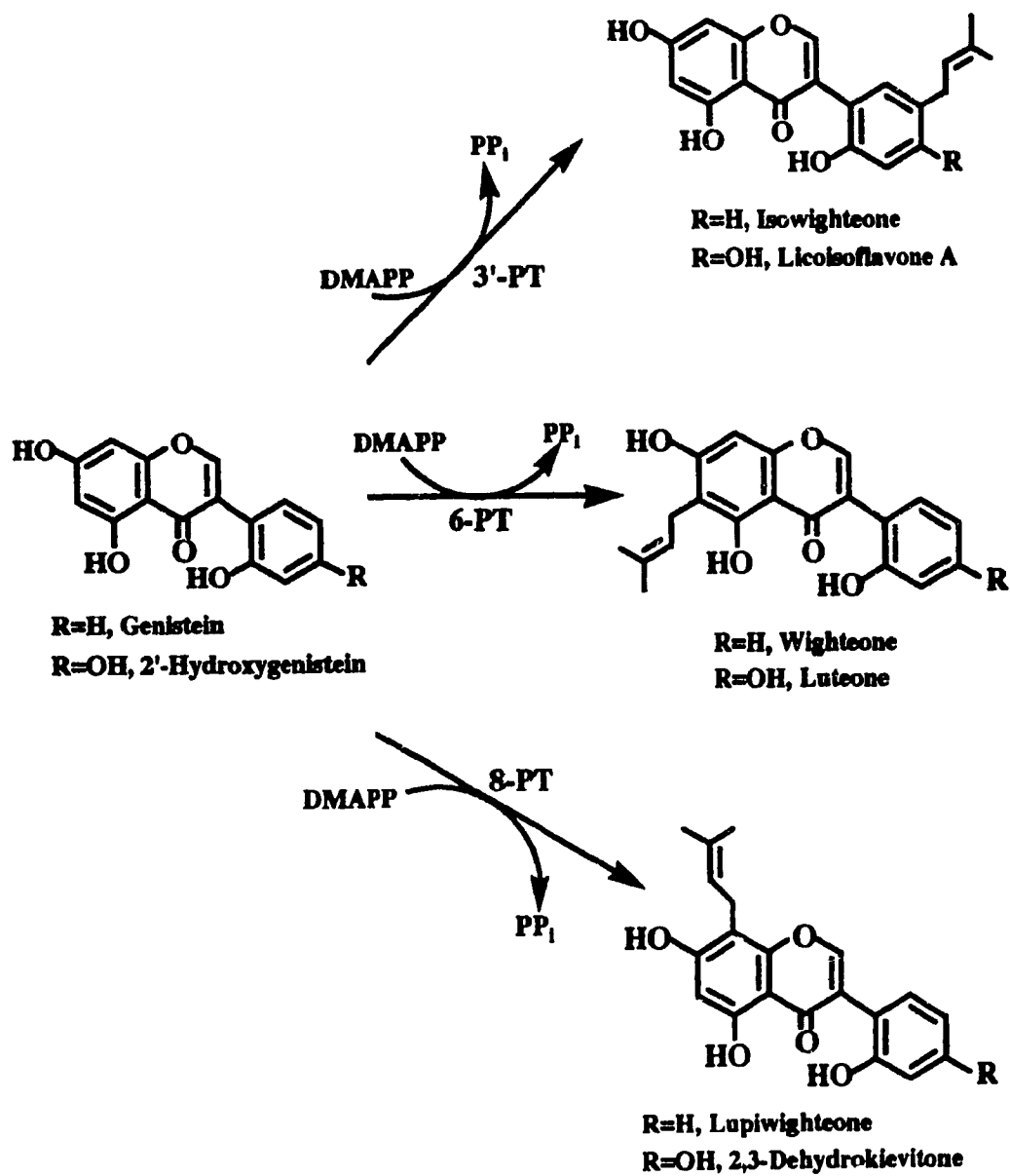


Table 3. Isoflavone prenyltransferase activity in different fractions isolated from tissue homogenates¹.

Enzyme Preparation ²	IFPT Activity (dpm/mg protein)	
	Radicles	Cells
S ₈	7,000	3,100
S ₂₀₀	0	6,700
P ₂₀₀	27,700	15,900

¹Isoflavone prenyltransferase activity was assayed as described in the Methods section.

²S₈: supernatant following centrifugation at 8000g.

S₂₀₀: supernatant from centrifugation at 200,000g following MgCl₂ precipitation.

P₂₀₀: pellet from centrifugation at 200,000g following MgCl₂ precipitation.

Figure 6. Photograph of an autoradiogram of the chromatographed reaction products when microsomal preparations of *L. albus* radicles (lanes 1 and 3) and cell cultures (lanes 2 and 4) were assayed with genistein (lanes 1 and 2) and 2'-hydroxygenistein (lanes 3 and 4) as substrates. Co-chromatography with reference compounds was carried out on silica gel plates (60 F-254) using *n*-pentane-Et₂O-HOAc (15:5:1) as solvent. The designations 6-, 8- and 3'- refer to the prenylated positions of the reaction products. "o" denotes the origin.

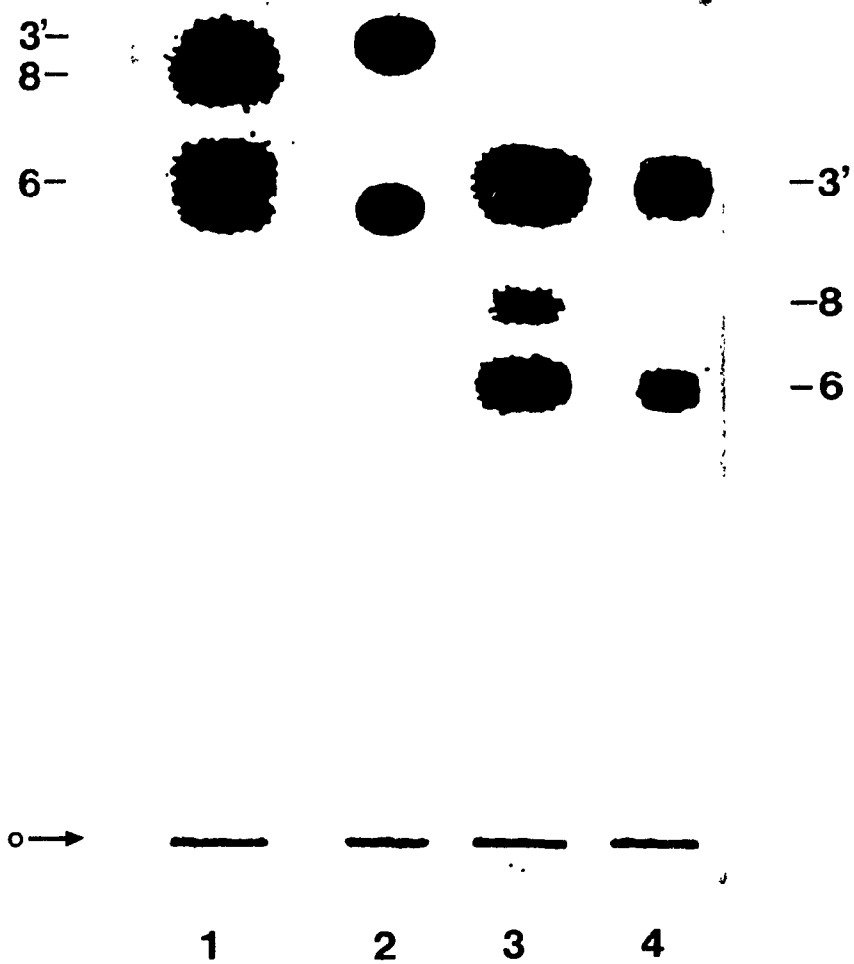


Figure 7. Photograph of an autoradiogram of the chromatographed reaction products when microsomal preparations of *L. albus* cell cultures were assayed with genistein (lane 1) and isowighteone (lane 2) as substrates. Co-chromatography with reference compounds was carried out on silica plates (60 F-254) using *n*-pentane-Et₂O-HOAc (15:5:1) as solvent. The designations 6-, 3'- and 3',5'- refer to the prenylated positions of the reaction products. "o" denotes the origin.

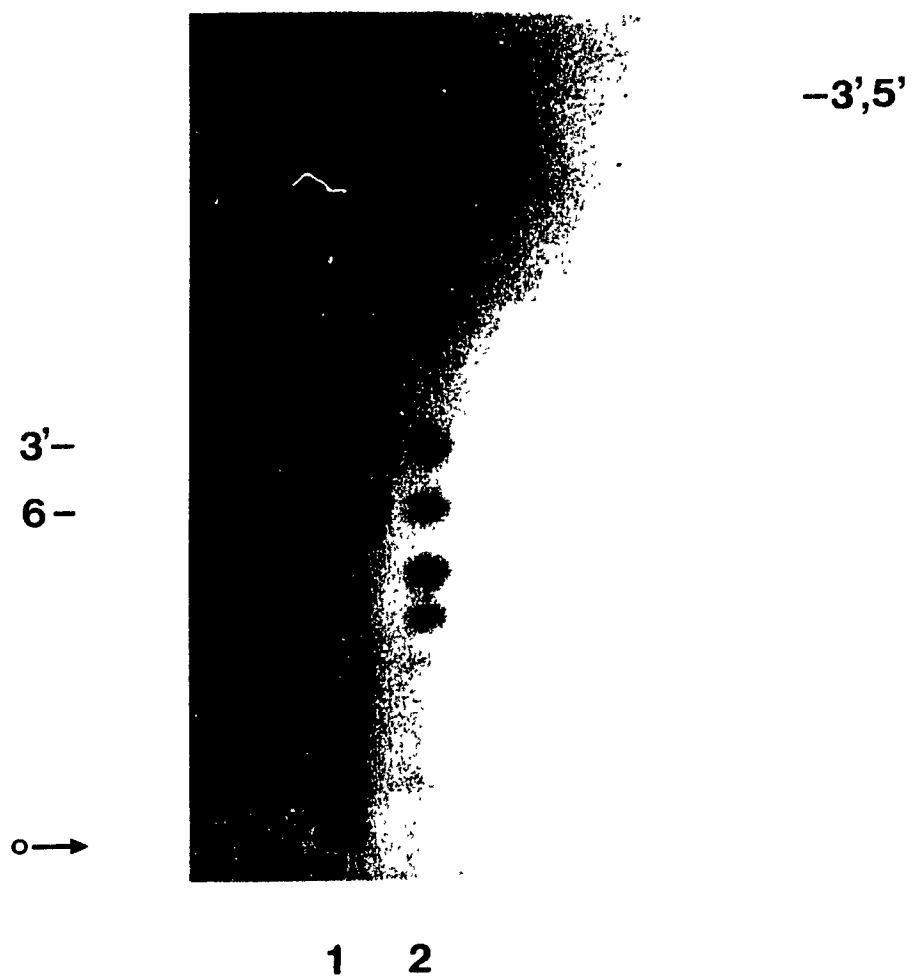
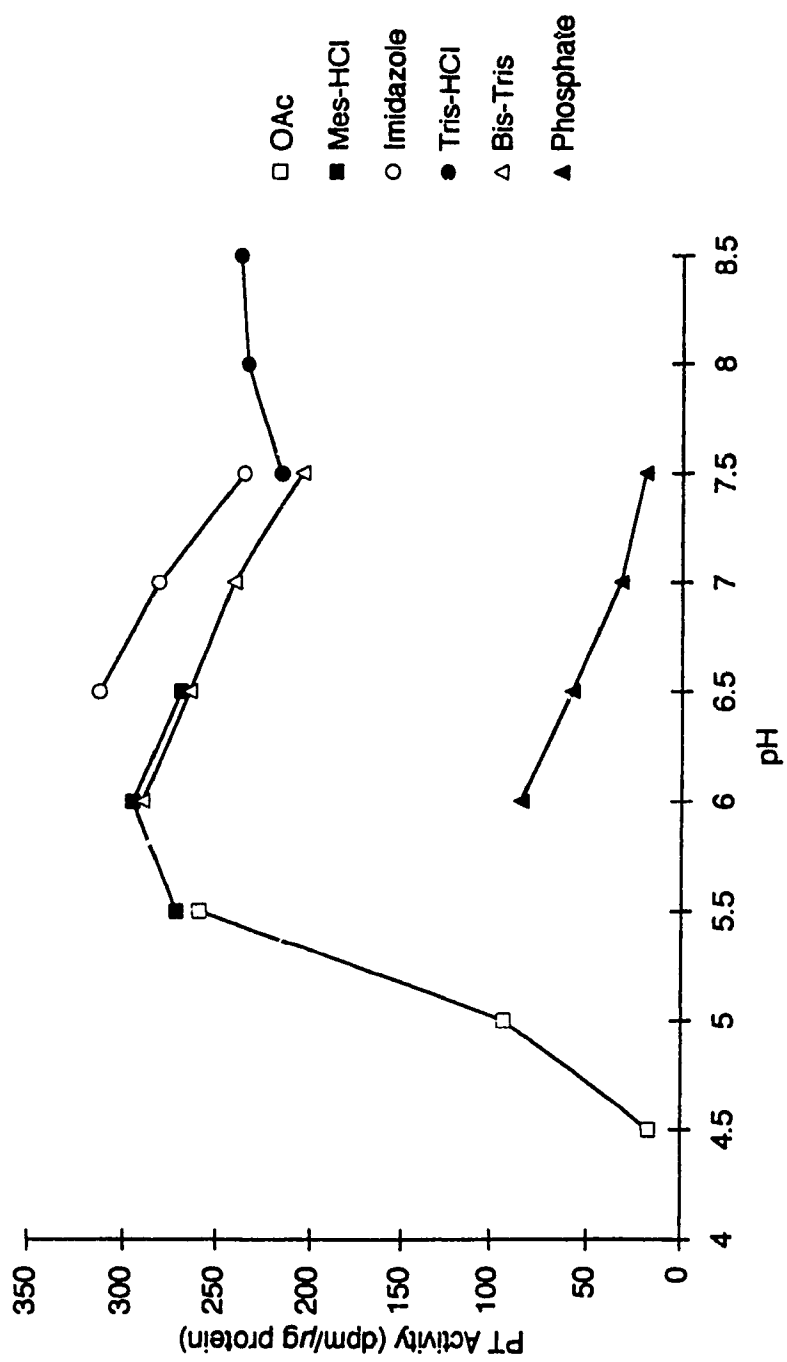


Figure 8. pH optimum for prenyltransferase activity using genistein as substrate. Twenty-five μL of the microsomal pellet (20 μg protein) was added to 100 μL of assay mixture and assayed for enzyme activity. Buffer concentrations in the assay mixture were 200 mM.



E.1.3. Linearity with protein concentration

At pH 7.5, the enzyme reaction rate was linear with the amount of protein added up to 15 μg of the microsomal preparation (equivalent to 150 $\mu\text{g}/\text{ml}$ protein) (Fig. 9) using the standard assay conditions.

E.1.4. Linearity with time

At 30°C, the IFPT-catalyzed reaction was linear with time up to 60 min (Fig. 10) using the standard assay conditions.

E.1.5. Kinetic properties

Apparent K_m values of 3.4 μM and 5.1 μM were obtained for the substrates genistein and 2'-hydroxygenistein, respectively, as determined by Lineweaver-Burk plots (Fig. 11, a and b). An apparent K_m of 3.6 μM was obtained for DMAPP, the prenyl donor (Fig. 11, c).

E.1.6. Effect of IPP, a specific inhibitor

Isopentenyl pyrophosphate (IPP), a competitive inhibitor of prenyltransferases, inhibited isoflavone prenyltransferase activity with an estimated K_i of 5.0 μM (based on 50% inhibition of PT activity)(Fig. 12). DOM-solubilized prenyltransferase activity was inhibited by 50 % at an IPP concentration of 8.0 μM , as compared with that of the buffer control (in absence of DOM) which was inhibited by 50% at an IPP concentration of 11.0 μM . On the other hand, the remaining

Figure 9. Linearity of the prenylation reaction using genistein as substrate in the presence of different amounts of microsomal protein under the standard assay conditions.

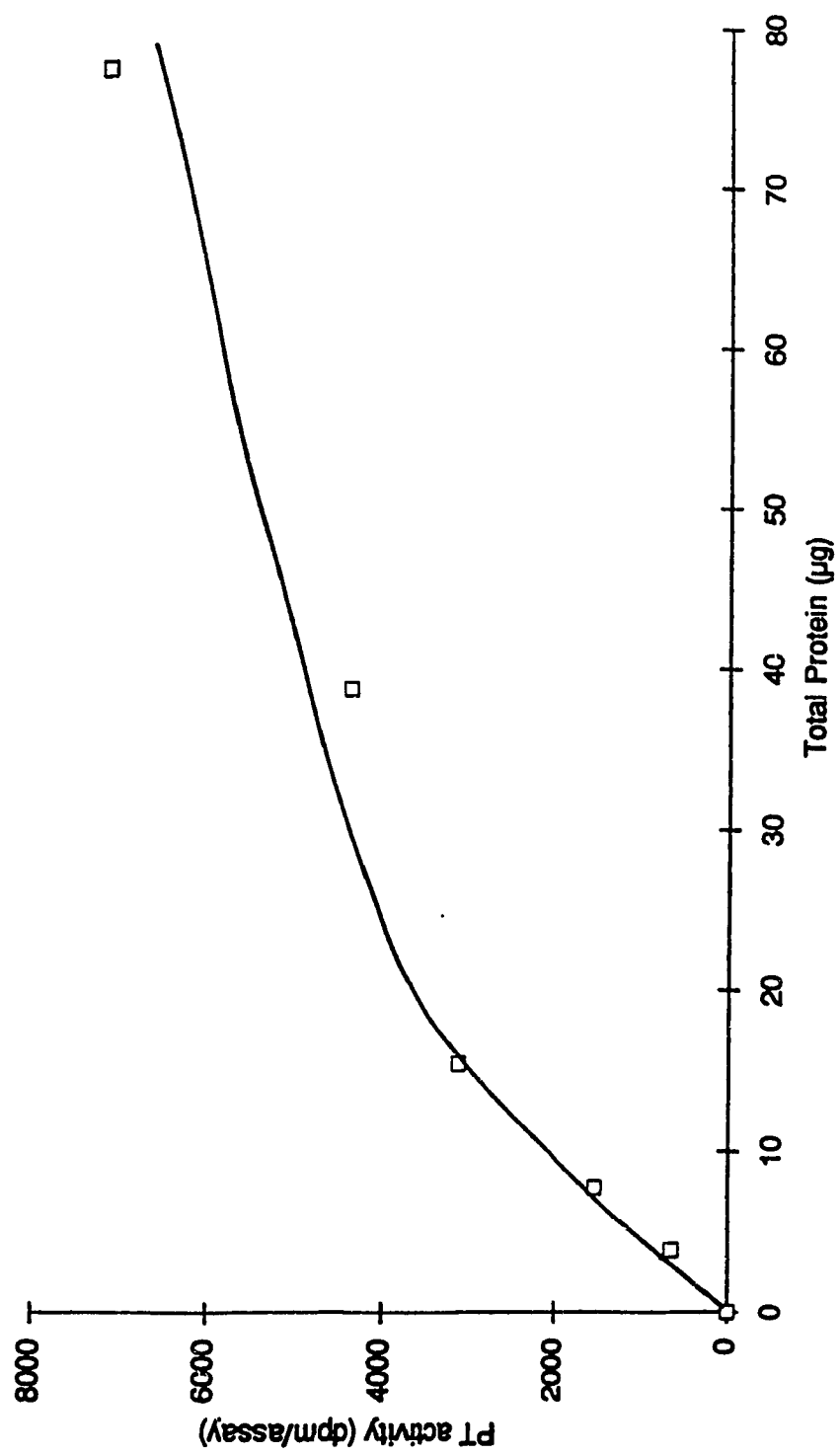


Figure 10. Linearity of prenylation reaction, using 5.5 μg of microsomal protein and genistein as substrate, for various incubation times under the standard assay conditions.

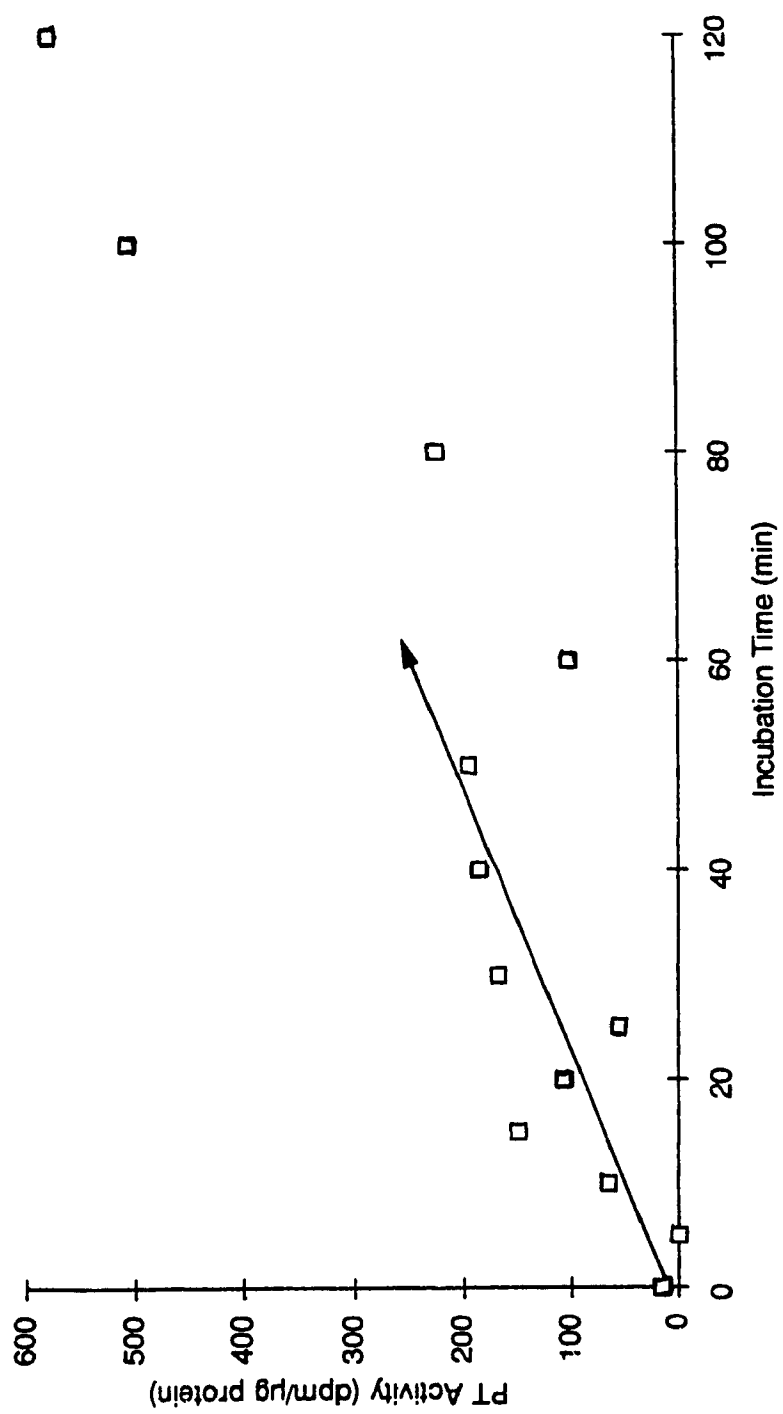


Figure 11. Double-reciprocal plots of initial velocities of IFPT activity with genistein (A), 2'-hydroxy-genistein (B) and DMAPP (C) as substrates.

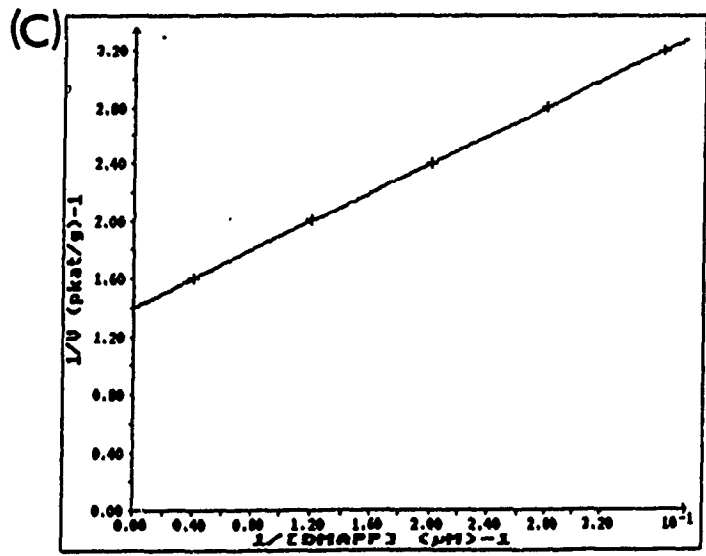
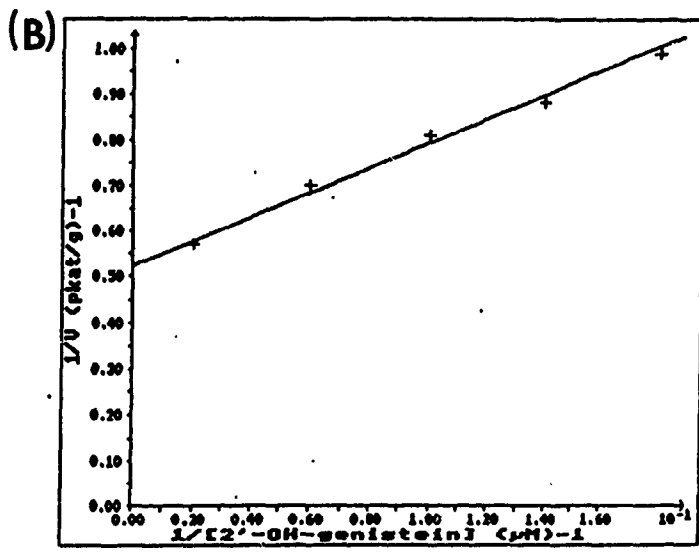
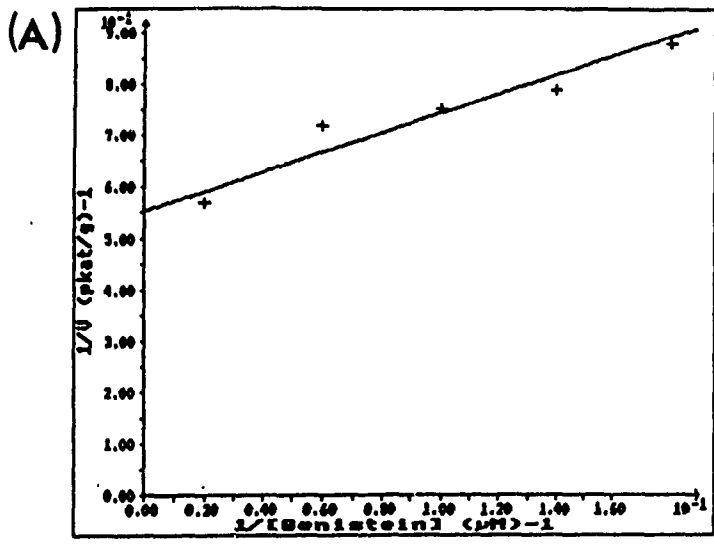
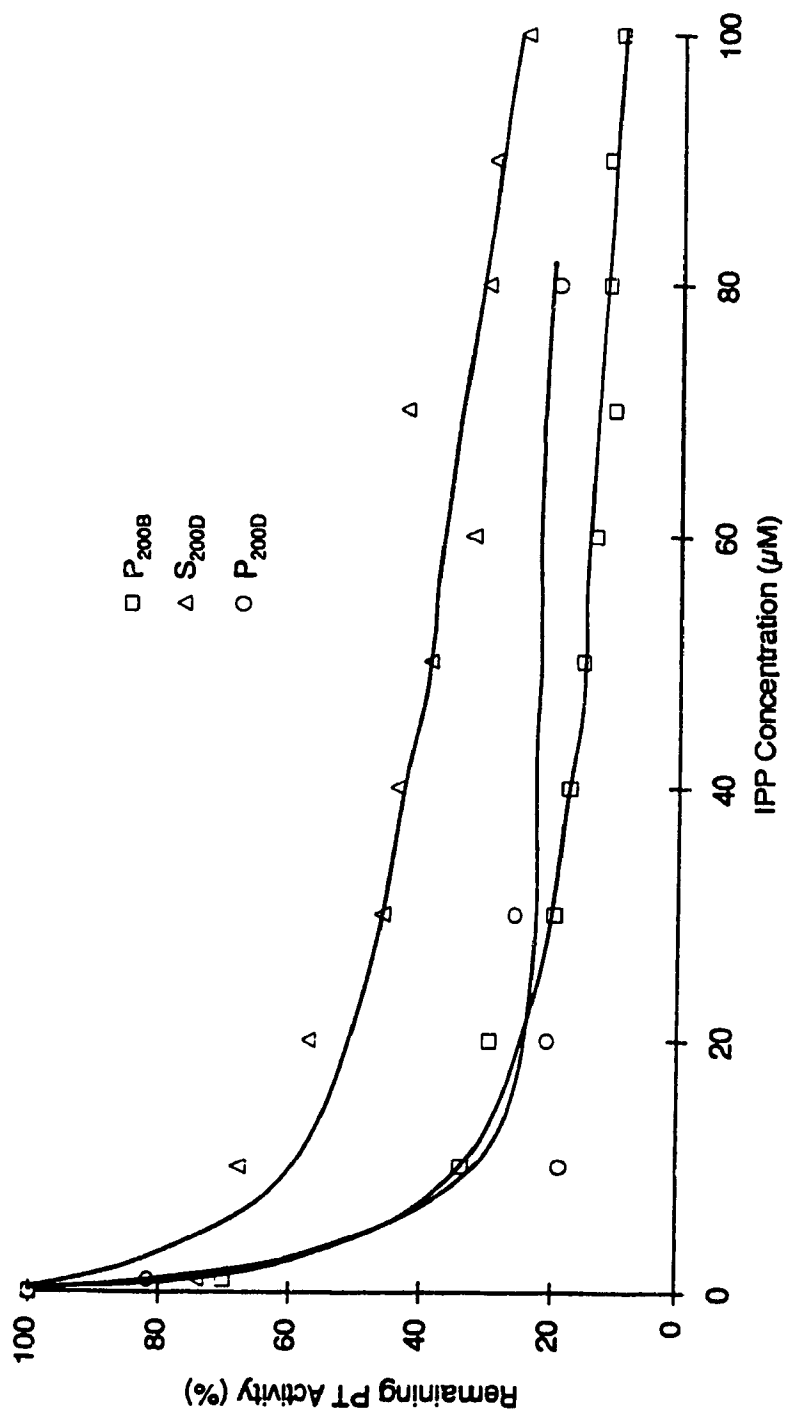


Figure 12. Effect of isopentenyl pyrophosphate (IPP) on DOM-solubilized (Δ) and non-solubilized (\circ) prenyltransferase activity. The buffer control (\square) represents microsomal pellets treated with buffer instead of DOM. PT activity with 0 μ M IPP was taken as 100%.



residual activity in the DOM-treated pellet (non-solubilized activity) was shown to be inhibited by 50% at an IPP concentration of 3.0 μM (Fig. 12).

E.1.7. Effect of divalent cations

Using the standard assay conditions, prenyltransferase activity exhibited a preference for Mn^{2+} (at 12 mM) over Co^{2+} for optimal activity (Table 4). In the absence of divalent cations, PT activity was 13% of that obtained in the presence of 12 mM Mn^{2+} . On the other hand, the PT activity observed in the presence of 12 mM Co^{2+} or 1.2 mM Mn^{2+} was 59% of that observed in the presence of 12 mM Mn^{2+} , and 11% at 1.2 mM Co^{2+} .

E.1.8. Substrate specificity

Microsomal preparations from *L. albus* radicles and cell suspension cultures displayed a marked preference for the isoflavones genistein and its 2'-hydroxy derivative as the prenyl acceptors, as compared with other flavonoids (Table 5). Daidzein, a 5-deoxyisoflavone, was also accepted (to approx. 50% of genistein), although the position of prenylation has not been determined due to lack of reference compounds. On the other hand, the isoflavones formononetin and piscisoflavone B were not accepted as substrates, and neither were naringenin (flavanone), luteolin (flavone) or quercetin (flavonol).

Table 4. Effect of divalent cations on isoflavone prenyltransferase activity¹.

Cation	Concentration (mM)	Relative Activity (%)
None	--	13
Mn ²⁺	12.0	100
Co ²⁺	12.0	59
Mn ²⁺	1.2	59
Co ²⁺	1.2	11

¹The standard assay was used with the microsomal pellet protein and genistein as substrate, as described in the Methods section.

The highest enzyme activity obtained was considered 100%, and amounted to 108,202 dpm/mg protein.

Table 5. Substrate specificity of *L. albus* prenyltransferase¹.

Substrate	Flavonoid Class	Relative Activity ² (%)
Genistein	Isoflavone	100
2'-Hydroxygenistein	Isoflavone	100
Daidzein	5-Deoxyisoflavone	47
Formononetin	Isoflavone	6
Piscisoflavone B	Isoflavone	2
Naringenin	Flavanone	5
Luteolin	Flavone	14
Quercetin	Flavonol	11

¹The standard assay was used with the microsomal pellet (protein) as described in the Methods section. The concentration of all substrates used was 40 μ M.

²Highest enzyme activity was set to 100%, and amounted to 287,358 dpm/mg protein.

E. 2. Prenyltransferase Activity of Different Organs/Tissues

Enzyme assays of microsomal pellets prepared from 12 day-old leaves, hypocotyls and radicles showed that radicles had 50-fold higher total prenylating activity than the hypocotyls and 10-fold higher than leaves (Table 6). Fine cells from 14 day-old cell suspension cultures exhibited 33% higher enzyme activity than their respective cell clumps (Table 7).

E. 2.1. Stability of prenyltransferase activity from different tissues

Prenyltransferase activity in microsomal pellets derived from 14 day-old cell clumps exhibited an increase in activity after storage for 24 hr at 4 °C while the activity from 19 day-old radicles and 14 day-old fine cells decreased by approximately 30% (Fig. 13). Enzyme activities prepared from either the radicles or fine cells decreased by 40% when stored for 48 hr at 4 °C. Approximately 70% of the enzyme activity of all the three tissues was lost after 72 hr storage at 4°C .

Attempts to stabilize the IFPT activity by increasing the amount of glycerol in the buffers (from 10% to 20% (v/v)) or by the addition of BSA, $MnCl_2$ or phospholipids to the proteins during column chromatography, were unsuccessful.

E.2.2. Time course of prenyltransferase activity during growth

Seeds of *L. albus* (cv. Primovski) were germinated as described in the methods section. The seedlings were then

Table 6: Prenyltransferase activity in different organs¹.

Organ ²	Relative Activity ³ (%)
Radicles ³	100
Hypocotyls ³	2
Leaves ³	8

¹The standard assay was used with the microsomal pellet protein and genistein as substrate, as described in the Methods.

²Organs were secured from 12 day-old seedlings.

³Highest enzyme activity was set to 100%, and amounted to 1.24×10^6 dpm/mg protein.

Table 7: Prenyltransferase activity of cell suspension cultures¹.

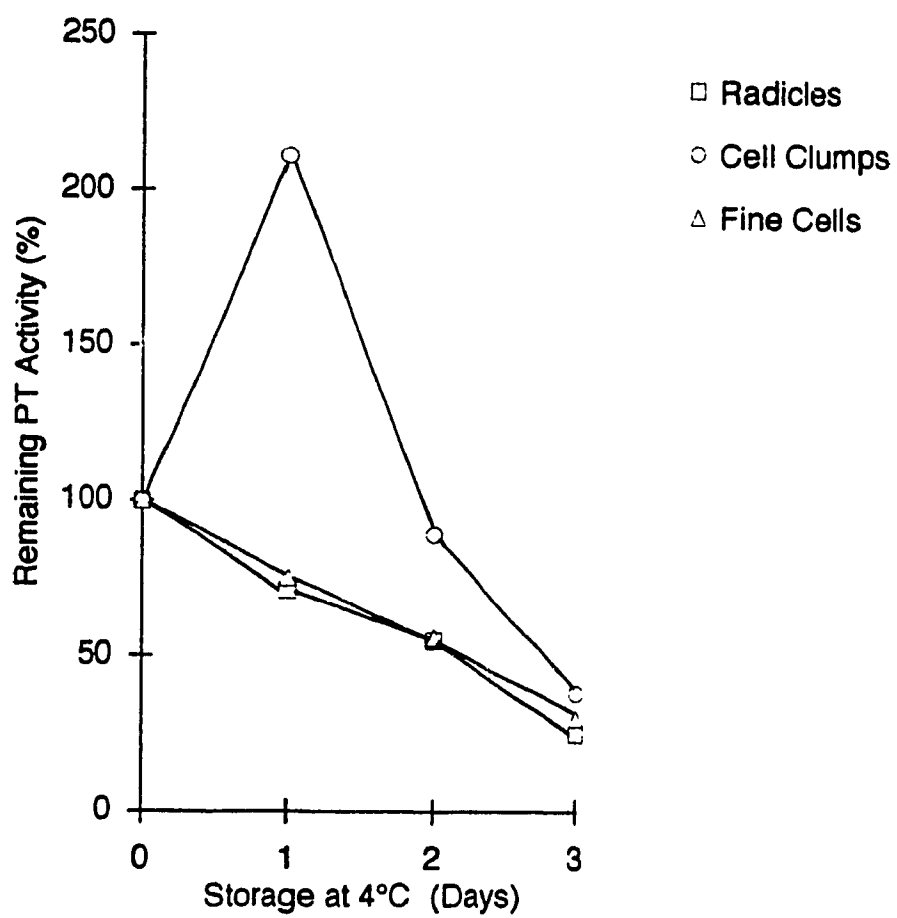
Tissue ²	Relative Activity ³ (%)
Fine cells	100
Cell clumps	66

¹The standard assay was used with the microsomal pellet protein and genistein as substrate, as described in the Methods.

²Tissue used was from 14 day-old cell suspension cultures.

³Highest enzyme activity was set to 100%, and amounted to 549,000 dpm/mg protein.

Figure 13. Stability of prenyltransferase activity in microsomal pellets, isolated from 19 day old radicles (\square) and 14 day old cell clumps (O) and fine cells (Δ), at 4°C. Twenty μL of microsomal preparation (13 μg protein) was assayed using genistein as substrate and the standard assay conditions.



separated into two groups, one to be inoculated and the other to serve as control. Ten day-old radicles were inoculated with *Rhizobium lupini* (Labon Inc., Boucherville, Qué.) and transferred to soil. Seedlings were harvested at 3- to 5-day intervals. Microsomal pellets prepared from radicles ranging in age from 3 to 50 days old showed that the highest prenyltransferase activity was found in 14 day-old radicles (Fig.14).

E.3. Optimal Conditions for Solubilization of Enzyme Activity

Various concentrations of different detergents were used in an attempt to solubilize the prenyltransferase activity from its membrane environment (Table 8). Of the different detergents tested, DOM (a nonionic detergent of the alkyl glucoside type) solubilized 81% of the total activity in microsomal preparations prepared from cultured cells and 66% of the activity from radicles. Further experiments indicated that DOM could be used over a wide range of protein:detergent ratios at an optimal concentration of 2% (w/v), where 80% of the total activity could be solubilized from microsomal pellets of cultured cells (Fig. 15).

E.3.1. Product ratios

The ratios of the enzyme reaction (monoprenylated) products varied with respect to the tissue, as well as with the detergent used to solubilize the prenyltransferase

Figure 14. The effect of inoculation of *L. albus* radicles with *Rhizobium lupini* on IFPT activity.

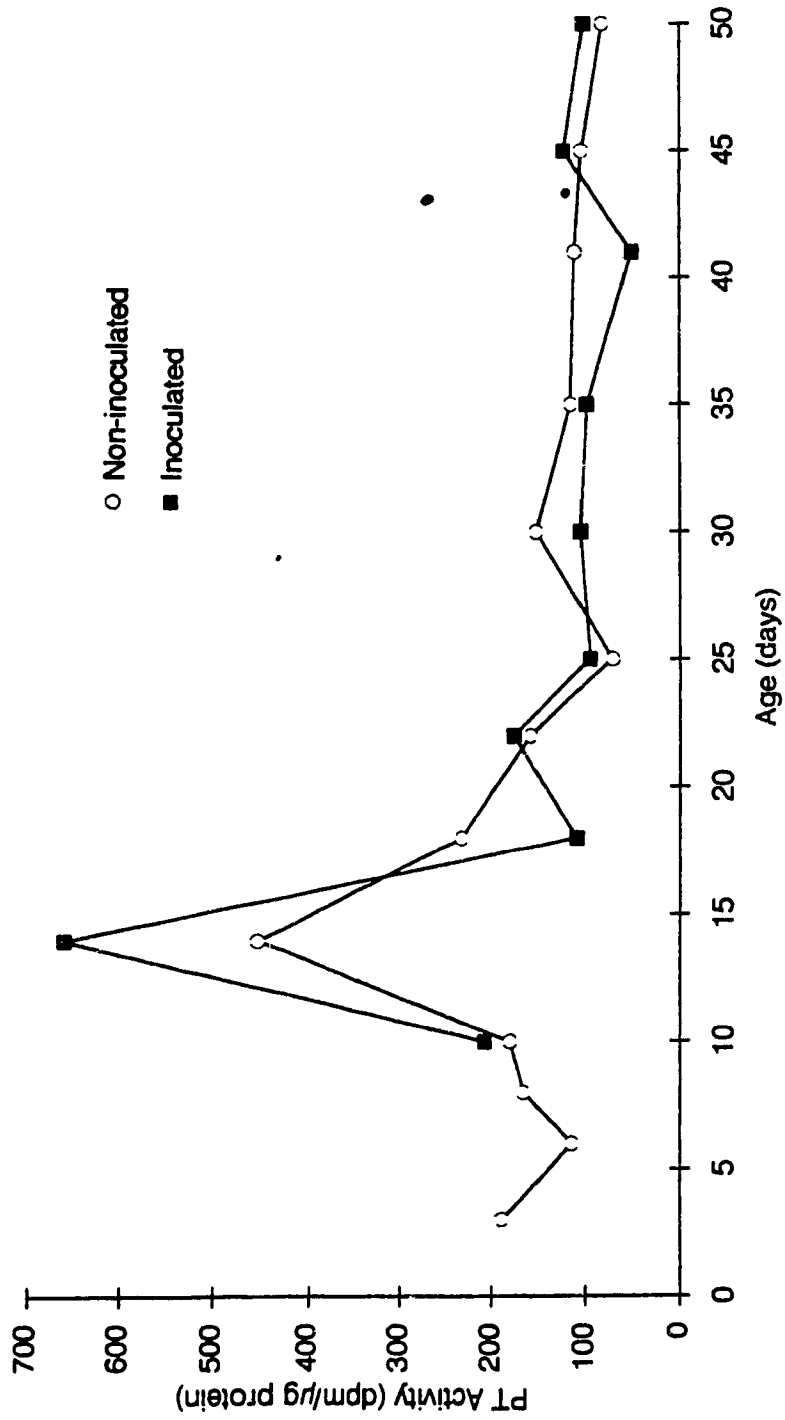


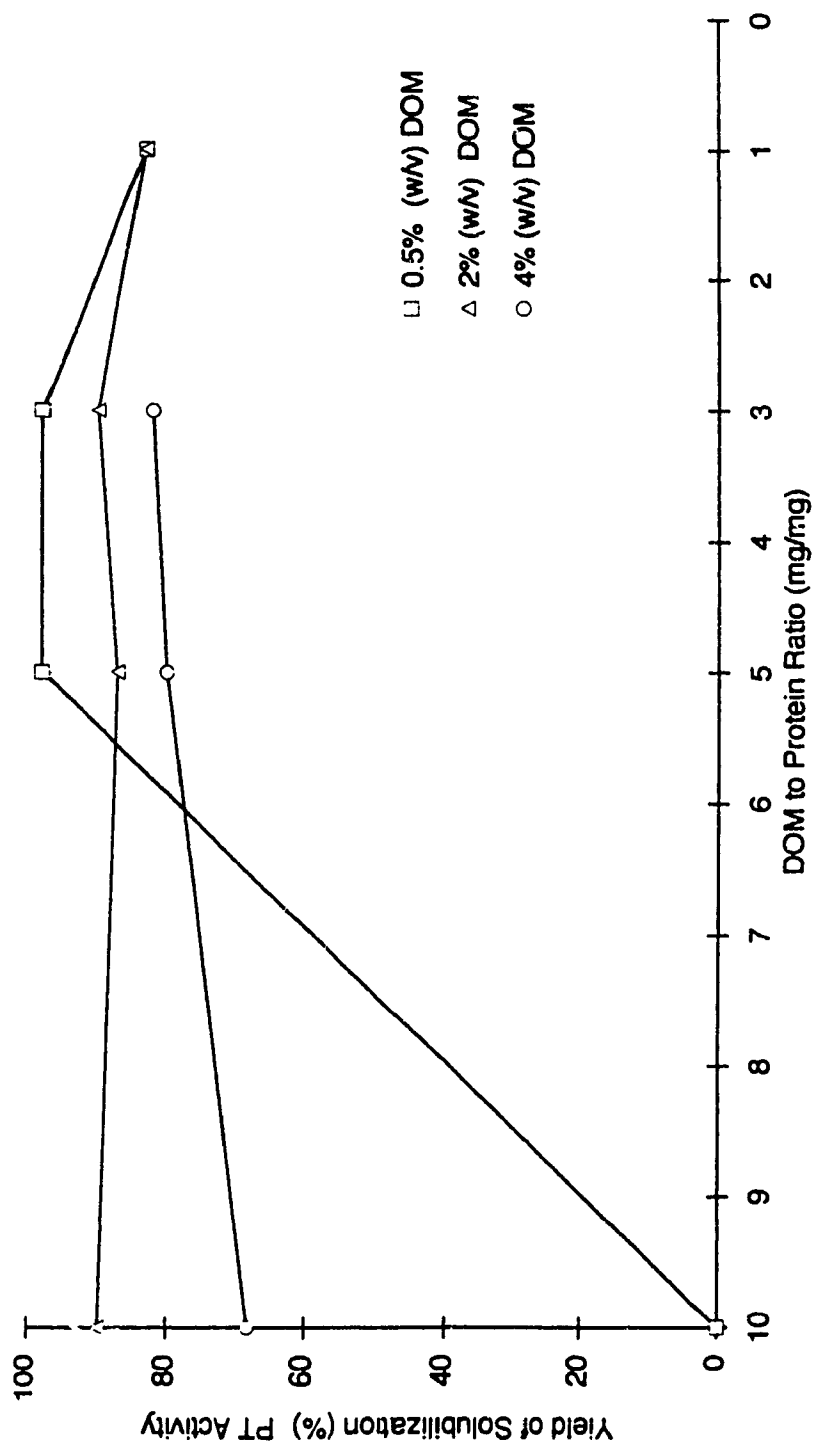
Table 8. Solubilization of prenyltransferase activity from lupin membrane pellets with different detergents.

Treatment	% Activity ¹			
	Cells		Radicles	
	Supernatant	Pellet	Supernatant	Pellet
Buffer (control)	6	94	18	82
Chaps ²	20	80	33	67
DOM ²	81	19	66	34
Nonidet P-40 ²	43	57	51	49
Triton X-100 ²	33	67	--	--

¹Enzyme activities of microsomal preparations from radicles and cell cultures varied between 4200-6800 and 18,500-115,000 dpm/mg protein with genistein as substrate; 6000-43,000 and 8800-350,000 dpm/mg protein with 2'-hydroxygenistein, respectively.

²Concentrations of detergents used were: 0.75% (w/v) Chaps, 2%(w/v) DOM, 0.2% (v/v) Nonidet P-40 and 0.1% (v/v) Triton X-100.

Figure 15. Effect of DOM to protein ratio on solubilization of prenyltransferase activity with increasing concentrations of DOM. (\square) 0.5% (w/v) DOM, (Δ) 2.0% (w/v) DOM, (\circ) 4.0% (w/v) DOM.



activity (Table 9). In cultured cells, the ratio of the 6- to 3'-prenylated isoflavones was 2:1 in DOM-treated pellets, as compared with a ratio of 1:3 in Nonidet solubilized pellets, and 1:1 in the buffer controls. On the other hand, in radicles, the ratio of the 6- to 3'-prenylated isoflavones was 1:1 in both DOM- and Nonidet-solubilized pellets, as compared with a ratio of 1:2 in the buffer controls. The results also indicate that the 3'-prenylating activity in microsomes derived from either the radicles or cultured cells treated with Nonidet was relatively higher than either the 6- or 8-prenylating activities.

E.4. Localization of IFPT Activity

The fractionation of organelles isolated from white lupin radicles or cell suspension cultures was carried out using continuous (10% to 70% w/v) sucrose gradients (Fig. 16). The majority of IFPT activity was found in the region made up of 30% to 50% sucrose (Fig. 17). Examination of individual fractions within this region, under the light microscope at highest magnification, indicated the presence of organelles resembling proplastids (in radicle preparations) or underdeveloped chloroplasts (in cell suspension cultures). Isoflavone prenyltransferase activity sedimented above the 50% (w/v) sucrose layer using discontinuous sucrose gradients (Fig. 18).

Table 9. Product ratios of monoprenylated isoflavones obtained from DOM- and Nonidet-treated microsomal pellets of radicles and cultured cells¹.

Treatment	Ratios					
	Radicles			Cells		
	6-Pr	3'-Pr	8-Pr	6-Pr	3'-Pr	8-Pr
Buffer (control)						
Supernatant	1	2	1	1	1	trace
Pellet	2	6	1	1	1	trace
DOM ²						
Supernatant	1	1	1	2	1	trace
Pellet	2	2	1	2	1	trace
Nonidet P-40 ²						
Supernatant	1	1	2	1	3	trace
Pellet	1	3	1	1	3	trace

¹The radioactive spots corresponding to each reaction product were scraped off the TLC plates, counted for radioactivity, and expressed as relative ratios. Values ranged from 1000 to 8500 dpm per product above background.

²Concentrations of detergents used were: 2%(w/v) DOM and 0.2%(v/v) Nonidet P-40.

Figure 16. Photograph of a continuous sucrose gradient (10% to 70%, w/v). Arrow indicates the region where isoflavone prenyltransferase activity sedimented (30% to 50%, w/v sucrose).

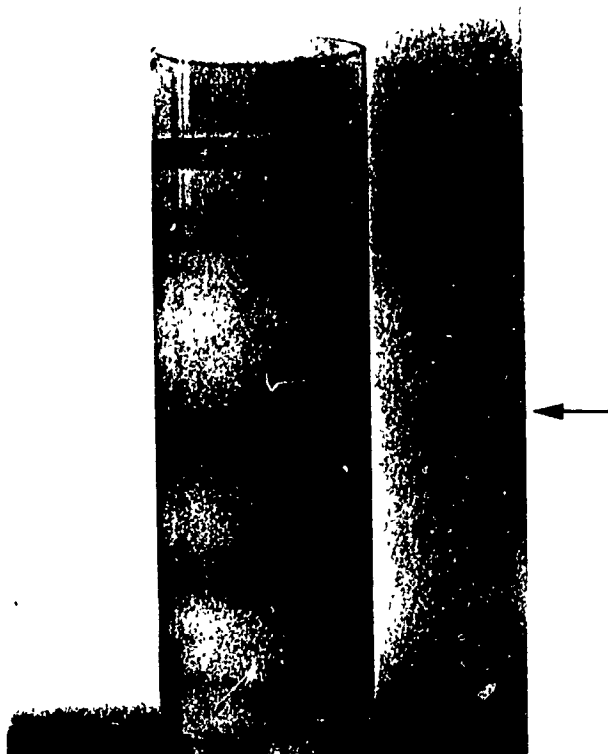


Figure 17. IFPT activity of different fractions from a *L. albus* radicle preparation separated on continuous sucrose gradients (10% to 70 %, w/v sucrose). Sucrose concentration (■), total IFPT activity (□).

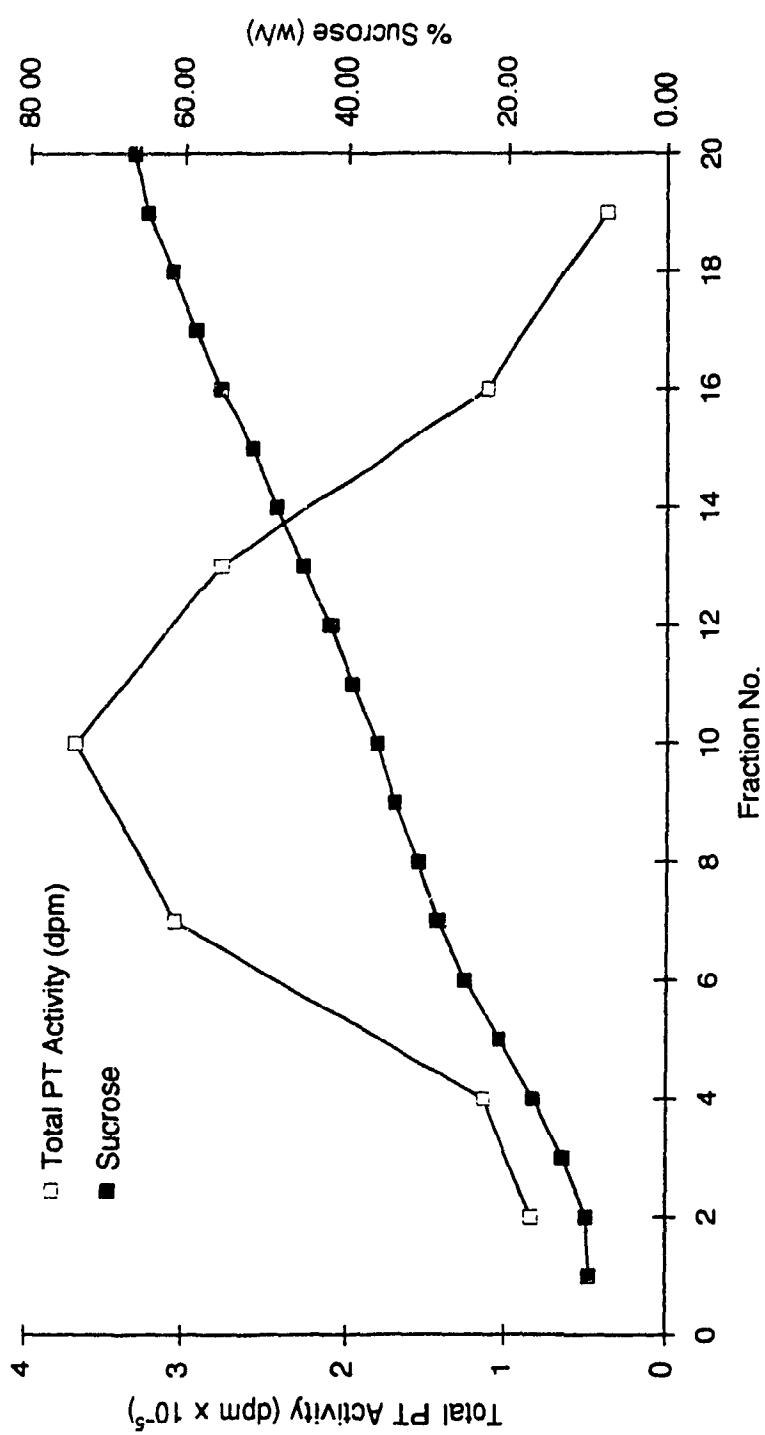
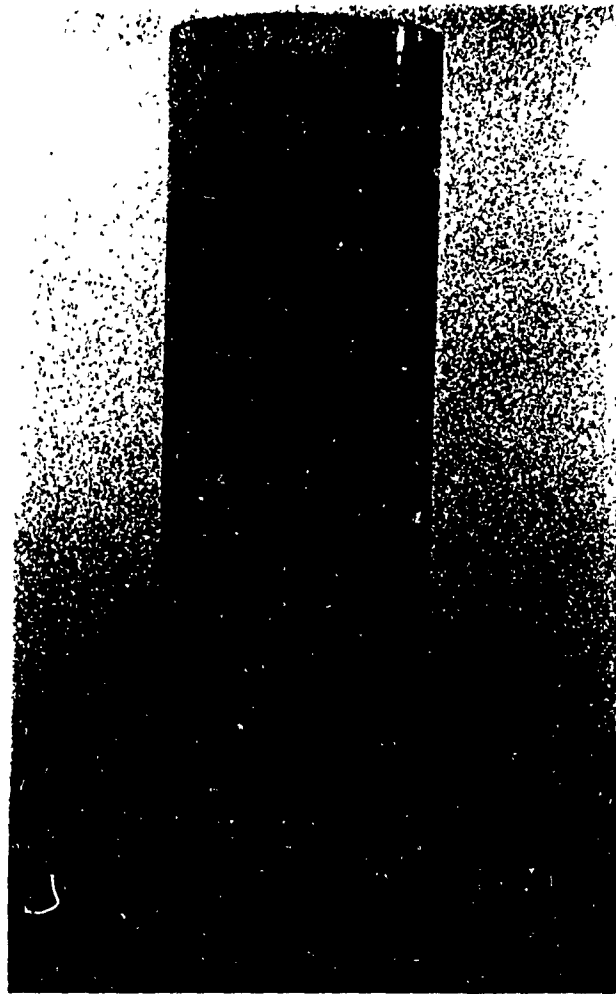


Figure 18. Photograph of discontinuous sucrose gradient (30%, 50%, 70%, w/v sucrose). Arrow indicates the band where IFPT activity sedimented.



E.5. Partial Purification of PT Activity

Successive chromatography of DOM-solubilized microsomal preparations on a Mono Q column, following Sephacryl S-400 (Fig. 19), resulted in a total loss of prenyltransferase activity. However, the enzyme was partially purified by chromatography of a microsomal preparation from cell cultures on Fractogel TMAE-EMD 650 (S) (Fig. 20), which allowed the partial separation of the 6-prenylating activity from the 3'-activity. Although the reaction products from the three peaks of activity displayed some cross-contamination, the autoradiographed products (Fig. 21) indicated the presence of distinct activities as confirmed by their respective product ratios (Table 10). The product ratio of 3'- to 6-monoprenyl genistein for the protein fraction with highest enzyme activity from peak I (Fig. 20, fraction #60) was determined to be 4:1 (Fig. 21, lane 1 and Table 10), as compared to 1:3 (Fig. 21, lane 2 and Table 10) for the activity recovered from peak II (Fig. 20, fraction #64) and 3:1 for peak III (Fig. 20, fraction #70 and Fig. 21, lane 3 and Table 10). An elution profile similar to that obtained with the Fractogel TMAE-EMD 650 (S) was reproduced when a DOM-solubilized microsomal preparation from cell suspension cultures was chromatographed on a Mono Q column (Fig. 22). However, the reaction products (Fig. 23) and their respective ratios (Table 11) indicated that the 6- and 3'- activities were not well resolved from each other.

Figure 19. Elution profile of prenyltransferase activity from Sephacryl S-400 column using genistein as substrate. The column was pre-equilibrated and the protein eluted with 20 mM Tris-HCl buffer, pH 7.5 containing 14 mM β -ME, 10% (v/v) glycerol and 0.1% (w/v) DOM. The molecular weight of the enzyme was determined using a similar column and the following protein standards: V_0 , blue dextran (2,000 kDa); THY, thyroglobulin (669 kDa); APF, apoferritin (443 kDa); β -AMY, β -amylase (200 kDa); ADH, alcohol dehydrogenase (150 kDa).

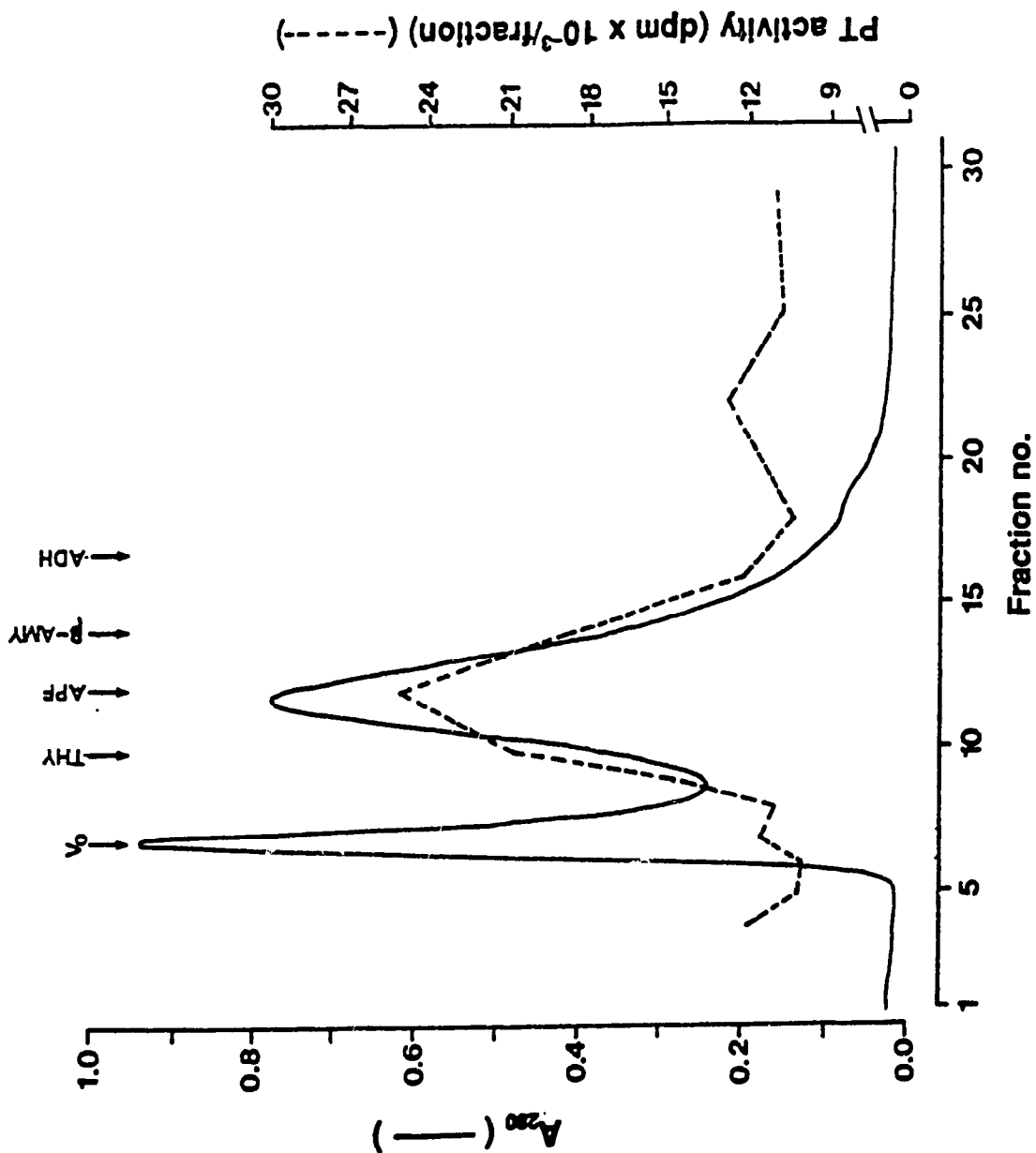


Figure 20. Elution profile of prenyltransferase activity from Fractogel TMAE-EMD 650 (S) column. The DOM-solubilized (2% w/v) microsomal pellet with a DOM:protein ratio of 8:1 (w/w) was applied to a column which had previously been pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5 containing 14 mM β -ME, 20% (v/v) glycerol, 5% (w/v) betaine and 0.04% (w/v) DOM. The protein was eluted using a gradient (0-250 mM) KCl in the same buffer, and fractions were assayed for 6- and 3'- activities using genistein as the substrate.

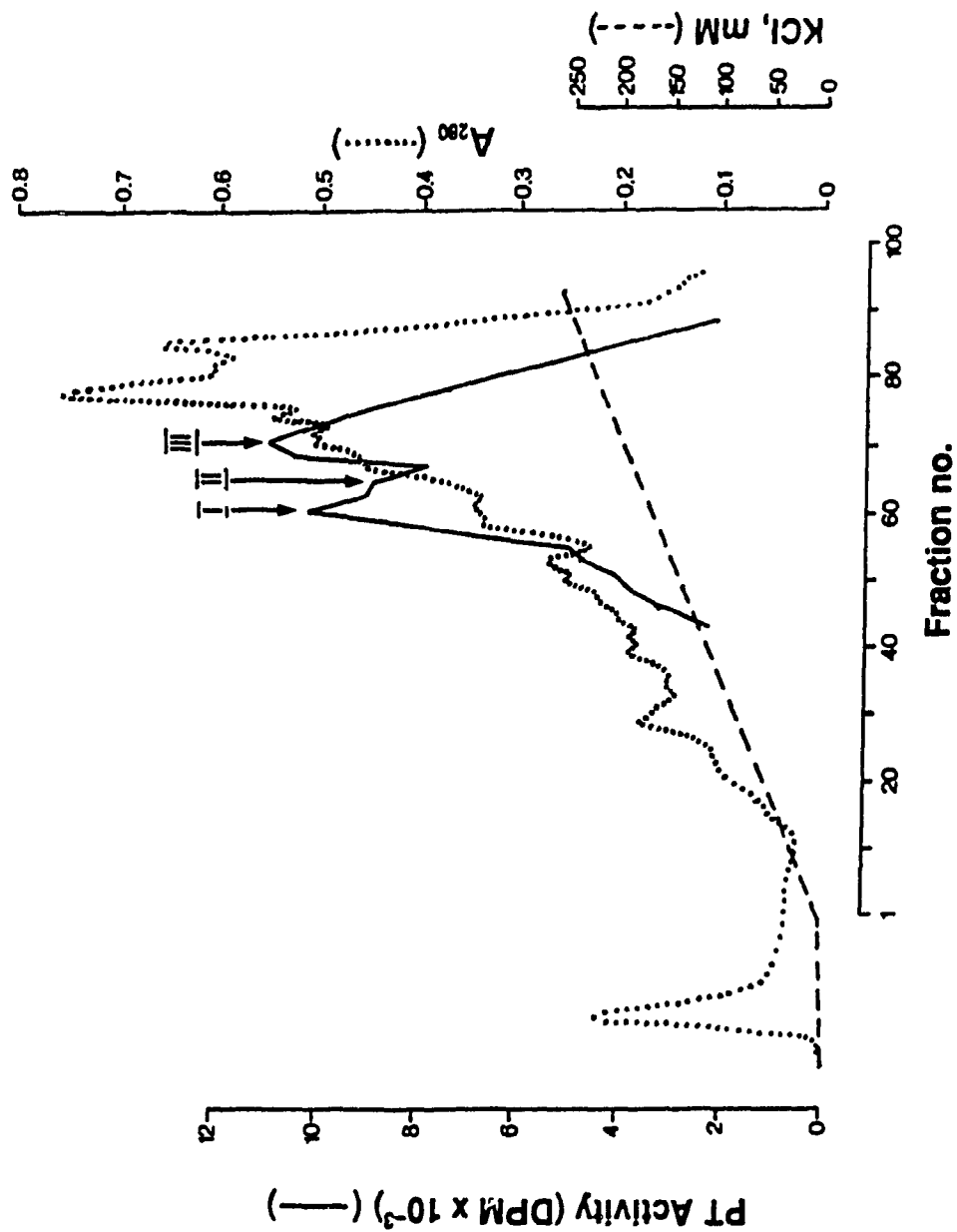


Figure 21. Photograph of an autoradiogram of the reaction products from the peak activity of fractions eluted from the Fractogel TMAE-EMD 650 (S) column using genistein as substrate. The products were co-chromatographed with reference compounds on silica gel TLC plates (60 F-254) using *n*-pentane-Et₂O-HOAc (15:5:1) as the solvent system, sprayed with ENHANCE and autoradiographed on X-ray film. Lanes 1, 2 and 3 represent the chromatographed reaction products from peak fractions I, II and III, respectively. o, origin; 6-, 6-monoprenyl genistein (wighteone); 3'-, 3'-monoprenyl genistein (isowighteone).

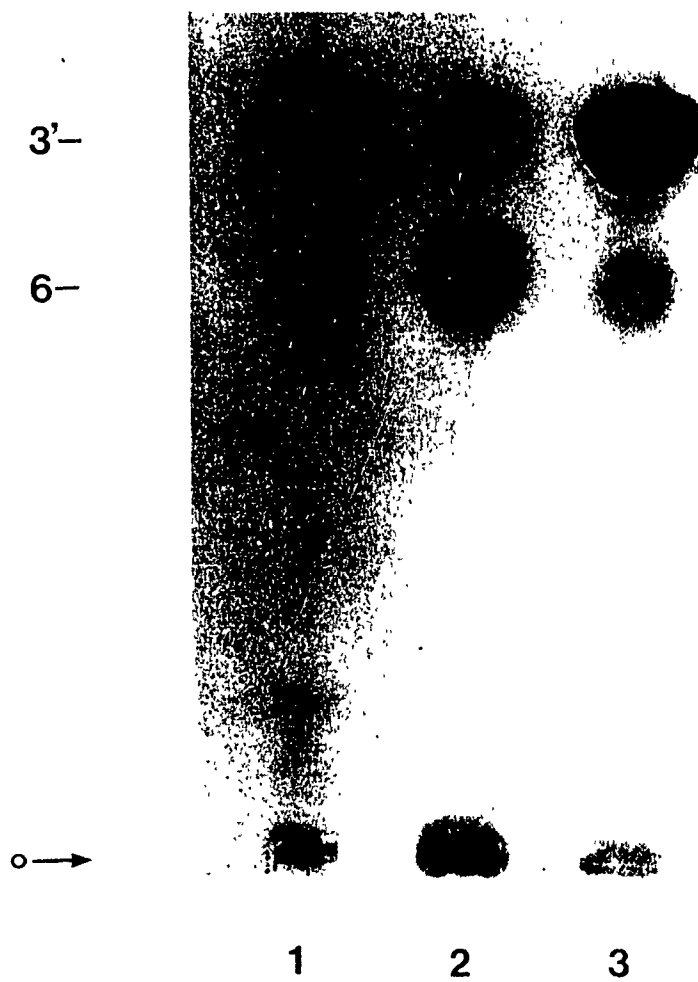


Table 10: Product ratios of the reaction products from the peak fractions eluted from Fractogel TMAE-EMD 650 (S) column¹.

Fraction #	Ratios	
	6-Pr	3'-Pr
60 (peak I) ²	1	4
64 (peak II)	3	1
70 (peak III)	1	3

¹The radioactive spots corresponding to each reaction product were scraped off the TLC plates, counted for radioactivity and expressed as relative ratios. Radioactivities ranged from 100 to 450 dpm per product above background.

²Peaks correspond to activity peaks shown in Fig. 20.

Figure 22. Elution profile of prenyltransferase activity from a Mono Q HR 5/5 column. The DOM-solubilized (2% (w/v) enzyme protein (DOM:protein ratio of 6:1, w/w) was applied to a column which had previously been pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5 containing 14 mM β -ME, 20% (v/v) glycerol, 5% (w/v) betaine and 0.04% (w/v) DOM. The protein was eluted using a gradient (0-250 mM) KCl in the same buffer, and fractions were assayed using genistein as substrate for the 6- and 3'- activities.

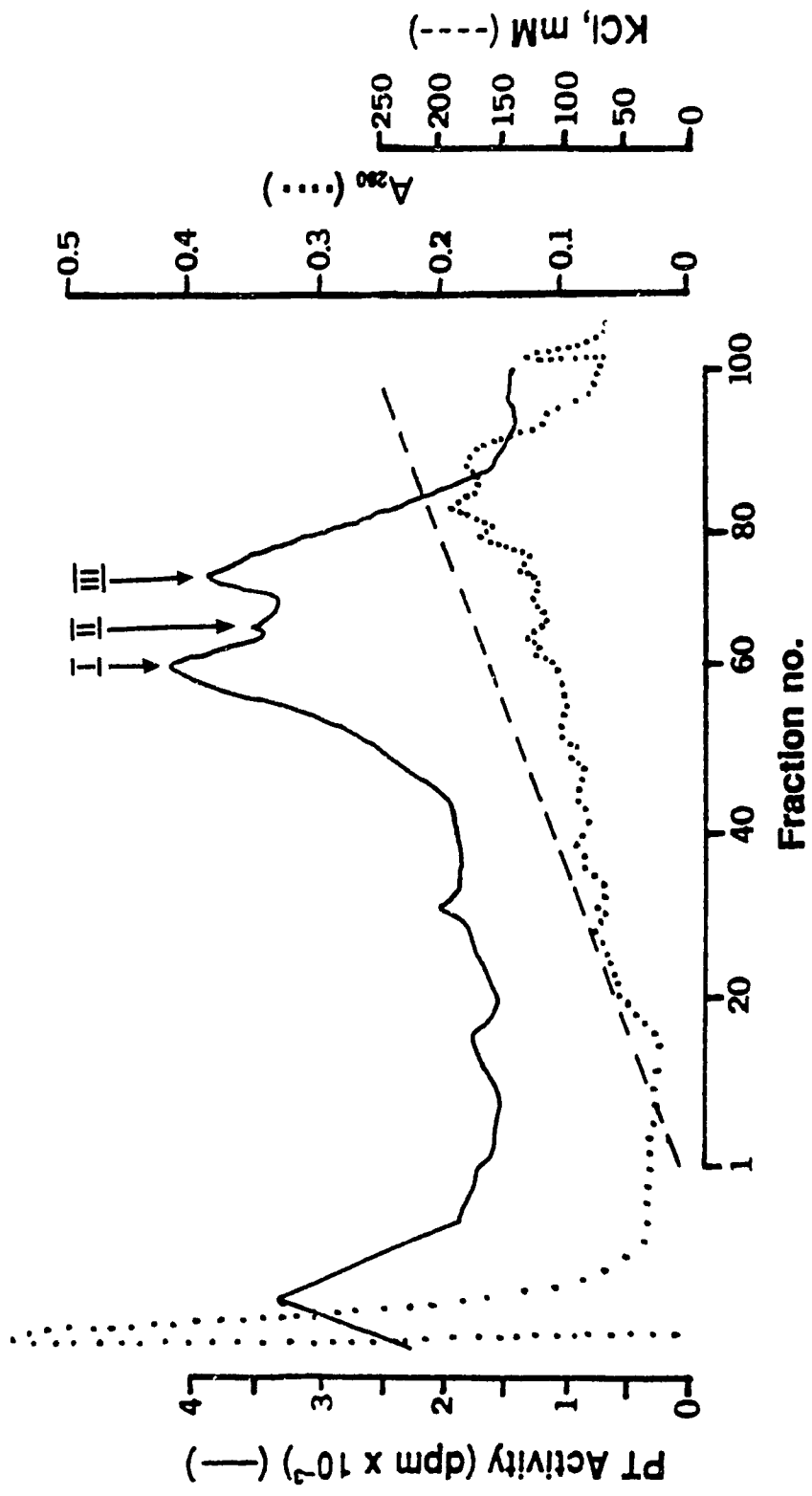


Figure 23. Photograph of an autoradiogram of the reaction products from the peak fractions eluted from Mono Q HR 5/5 using genistein as substrate. The products were co-chromatographed with reference compounds on silica gel TLC plates (60 F-254) using *n*-pentane-Et₂O-HOAc (15:5:1) as the solvent system, sprayed with ENHANCE and autoradiographed on X-ray film. Lanes 1, 2 and 3 represent the chromatographed reaction products from the three most active fractions of peaks I, II and III, respectively. (Peaks I, II and III refer to those activity peaks in Fig. 22). o, origin; 6-, 6-monoprenyl genistein (wighteone); 3'-, 3'-monoprenyl genistein (isowighteone).

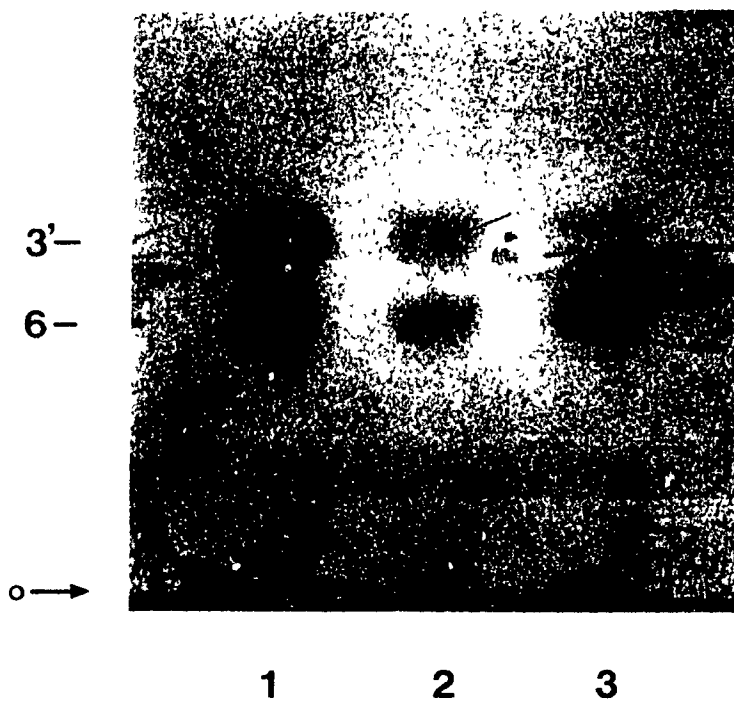


Table 11: Product ratios of the reaction products from the peak fractions eluted from Mono Q (HR 5/5)¹.

Fraction #	Ratios	
	6-Pr	3'-Pr
61, 62, 63 (peak I) ²	1	1
65, 66, 67 (peak II)	1	1.5
71, 72, 73 (peak III)	1	1

¹The radioactive spots corresponding to each reaction product were scraped off the TLC plate, counted for radioactivity and expressed as relative ratios. Radioactivities ranged from 100 to 420 dpm per product above background.

²Peaks correspond to activity peaks shown in Fig. 22.

E.6. Electrophoretic Protein Patterns

Electrophoresis of the eluted protein from both Fractogel and Mono Q columns was carried out under SDS-denaturing conditions (Fig. 24). The electrophoresed protein bands were revealed by staining with Coomassie blue. A number of proteins are visible indicating that the fractions have been only partially purified. Unfortunately the bands were only barely visible due to the low protein concentration.

Figure 24. Photograph of SDS-PAGE of the most active peak fractions eluted from Mono Q (Fig. 22) and Fractogel (Fig. 20) columns.

Lane 1: DOM-solubilized microsomal pellet prior to chromatography on Mono Q.

Lane 2: Peak I (Mono Q, Fig. 22).

Lane 3: Peak II (Mono Q, Fig. 22).

Lane 4: Peak III (Mono Q, Fig. 22).

Lane 5: Molecular weight standards. ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

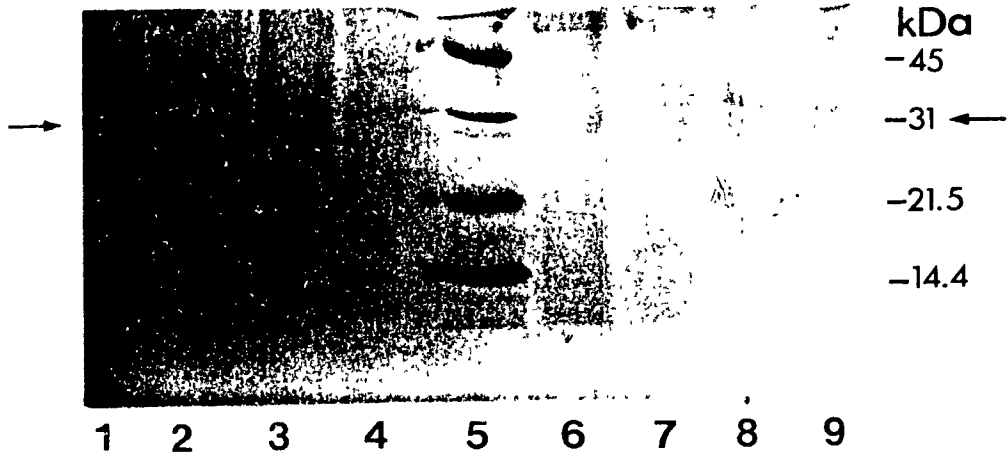
Lane 6: DOM-solubilized microsomal pellet prior to chromatography on Fractogel.

Lane 7: Peak I (Fractogel, Fig. 20).

Lane 8: Peak II (Fractogel, Fig. 20).

Lane 9: Peak III (Fractogel, Fig. 20).

Arrow indicates the region of the putative PT protein.



F. DISCUSSION

In contrast with other leguminous plants which prenylate isoflavonoid compounds in response to attack by microorganisms or to biotic/abiotic elicitors, white lupin has the remarkable ability to constitutively express the biosynthesis of a variety of prenylated isoflavonoids. These consist of 6-, 8-, and 3'-mono- as well as 6,3'-diprenylated derivatives of genistein and 2'-hydroxygenistein [Tahara et al., 1989]. Prenylated isoflavones and prenylated pterocarpanes have been proposed to act as antimicrobial metabolites [Harborne et al., 1976][Dixon et al., 1983], and are possibly involved as signalling molecules in *Rhizobium-Lupinus* symbiotic association [Gagnon, 1993].

The results reported here demonstrate the presence of distinct position-specific enzymes, in *L. albus* radicles and cell suspension cultures. These PTs catalyze the transfer of the prenyl group from dimethylallyl pyrophosphate (DMAPP) to positions 6-, 8- or 3'- of the naturally occurring isoflavones, genistein and 2'-hydroxygenistein (Fig. 6).

These prenyltransferases are specific to isoflavones, since neither flavanones, flavones or flavonols were accepted as substrates for prenylation (although the 5-deoxyisoflavone, daidzein was accepted for prenylation albeit at a much lower rate (Table 5)).

To our knowledge, this is the first reported instance in

which enzymatic prenylation of isoflavonoids has been shown to be catalyzed by distinct, position-specific enzymes. This is similar to other substrate- and position-specific flavonoid conjugating enzymes, such as the glycosyltransferases [Latchinian *et al.*, 1987] and methyltransferases [De Luca & Ibrahim, 1985a; Khouri *et al.*, 1988a; Khouri & Ibrahim., 1987; Khouri *et al.*, 1988b] of *Chrysosplenium americanum*, and the sulfotransferases of *Flaveria* spp. [Varin, 1992]. Prenylation of pterocarpans (isoflavonoid-type metabolites), in elicitor-challenged soybean cell suspension cultures, has previously been reported to occur at positions 2 and 4 [Welle & Grisebach, 1991], although, it has yet to be demonstrated whether prenylation at both positions is catalyzed by one, or two distinct enzymes.

In contrast with the 6-prenyltransferase activity reported earlier from fungal-elicited lupin hypocotyls [Schröder *et al.*, 1979], the enzymatic prenylation of positions 6-, 8- and 3'- of genistein and 2'-hydroxygenistein, in white lupin, is constitutively expressed and occurs predominantly in both radicles (Table 6) and cell suspension cultures (Table 7). Furthermore, we have demonstrated that the isoflavone prenyltransferase activity is integrally bound to the membranes, since the bulk of enzyme activity is associated with the microsomal pellet (Table 3) and could only be released from its environment by the use of detergents (Table 8).

Preliminary evidence presented here suggests that lupin IFPT activity is associated with the proplastids in radicles, and with the less developed chloroplasts in cell suspension cultures. These results are similar to those recently reported for pterocarpan prenyltransferase activity from bean and soybean that were located in plastid envelope membranes [Biggs et al., 1990].

There is evidence to suggest that lupin IFPTs, and possibly the entire isoflavonoid pathway, occur *in vivo* in the form of a multienzyme aggregate integrally bound to membranes. It is interesting to note that the pathway leading to flavonoid biosynthesis [Stafford, 1990; Hrazdina, 1992, and references cited therein] and flavonol *O*-methylation and *O*-glucosylation [Ibrahim, 1992, and references cited therein] have been proposed to take place on a multienzyme aggregate. The enzymes catalyzing these reactions would be aligned in a linear order to allow for the efficient channelling of their metabolites. The fact that isoflavone synthase has been reported to be a microsomal enzyme [Kochs & Grisebach, 1986], and that lupin IFPTs are readily inactivated when solubilized with detergents corroborate with the above view. In addition, since IFPTs retain detectable amounts of their endogenous substrates, and yield different product ratios even after separation on the highest resolution chromatographic support (such as the tentacle ion-exchanger), strongly suggests that these enzymes are in the form of a multienzyme aggregate that

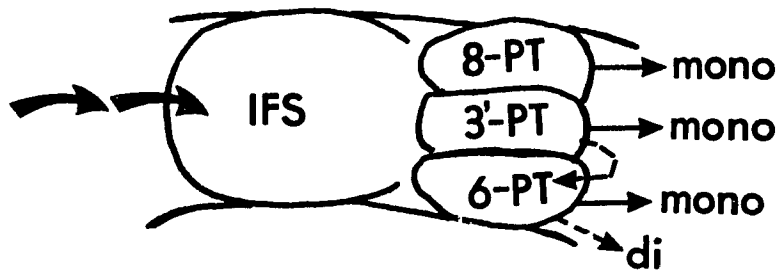
is integrally bound to their membrane environment, and are stabilized by their endogenous substrates. The sequence of enzymatic prenylation would thereby be determined by the arrangement of the individual IFPTs within the protein aggregate. For example, genistein would first be prenylated by the 3'-PT to its monoprenyl derivative, which in turn would be channelled to a vicinal 6-PT on the protein aggregate for diprenylation (Fig. 25). It would be expected, therefore, that upon disruption of the membrane-associated aggregate, the *in vivo* sequence of reactions be disrupted, and cannot be re-established *in vitro*.

White lupin accumulates lupalbigenin and 2'-hydroxy-lupalpigenin, the 6,3'-diprenylated derivatives of both genistein and 2'-hydroxygenistein [Tahara *et al.*, 1989]. It was considered of interest, therefore, to demonstrate the enzymatic reactions leading to the formation of diprenyl derivatives. The fact that only isowighteone (3'-prenyl genistein) was accepted for further prenylation and formed 3',5'-diprenylgenistein as the major product (Fig. 7), indicates a non-specific enzymatic prenylation due to disruption of the multienzyme aggregate *in vivo*. However, the fact that small amounts of the naturally occurring lupalbigenin was formed, suggests that 6,3'-diprenylated derivatives of genistein and 2'-hydroxygenistein are possibly synthesized from the 3'-monoprenyl rather than the 6-isomer. Plastid membranes and the endoplasmic reticulum have been

Figure 25. Proposed alignment of the enzymes, along a multi-enzyme aggregate, involved in the biosynthesis of isoflavones and their prenylation.

IFS, isoflavone synthase; mono, monoprenyl; di, diprenyl; 6-PT, 6-prenyltransferase; 8-PT, 8-prenyltransferase; 3'-PT, 3'-prenyltransferase.

(\longrightarrow) signifies monoprenylation, ($---\longrightarrow$) signifies diprenylation



proposed to be the likely sites for the biosynthesis of various secondary metabolites [Kleinig, 1989]. This suggests that the isoflavone biosynthetic pathway, including the prenylation reactions, may also take place on an integrally membrane-bound multienzyme aggregate, possibly located on the membrane envelope of plastids.

Membrane proteins can either be peripheral or integral (intrinsic). The former can be released from membranes by altering the ionic strength of the buffer, thereby disrupting the electrostatic interactions between the protein and the membrane. Intrinsic membrane proteins require amphiphilic reagents, such as detergents, which disrupt hydrophobic interactions [Capaldi & Green, 1972]. Detergents solubilize membrane proteins by displacing the lipids associated with the protein which, in turn, play an important role in maintaining the protein's native conformation. A consequence of the latter process is a decrease in enzymatic activity, resulting from partial unfolding of the protein by favouring one conformational state over the other, as was found with the reconstitution of torpedo acetylcholine receptor (AChR) [Criado *et al.*, 1984]. This receptor was shown to adopt a low- or a high-affinity for its substrate, depending on its conformational state as a result of the phospholipids used. It has also been suggested that one detergent may favour a particular conformation over another, which may explain the

different product ratios obtained with the lupin PT using different detergents (Table 9). Detergent binding sites on membrane proteins can display an expressed affinity for a specific polar head group of a detergent rather than its hydrophobic tail [Tanford & Reynolds, 1976] and, therefore, some detergents may be better than others at mimicking the protein's natural membrane environment. It is also conceivable that different conformations of the same protein may give rise to different product ratios.

In spite of the difficulties encountered in the solubilization of the membrane bound IFPT activity, and the instability of the enzyme preparation, the partial purification of DOM-solubilized IFPT activity from cell suspension cultures on Fractogel TMAE-EMD 650 (S) (a tentacle anion exchanger) resulted in the separation of the 3'- and 6-activities (Fig. 20). The latter support is an ion-exchanger with charged trimethyl amino ethyl groups positioned along an acrylic side chain support [Jansen, 1990], much like the suction cups along the tentacles of an octopus! The flexibility of this type of support allows retention of the native protein conformation, thus resulting in a better recovery of enzyme activity. Ion-exchangers, such as Mono Q, have charged groups linked to a rigid support; and in order for the protein to bind to the ion exchanger, the protein must undergo a conformational change which may cause loss of

enzymatic activity.

The product ratios obtained from the different activity peaks eluted from the tentacle column, clearly indicates the existence of distinct 3'- and 6- IFPT activities (Table 10). It is interesting to note that the product ratios of peaks I, II, and III indicate the presence of 3'-, 6- and 3'-PT activities, respectively. However, this repetitive pattern of activity for the 3'-PT is not unexpected to occur in *in vitro* conditions, due to disruption of the orderly *in vivo* sequence of these enzymes caused by the use of detergents. In fact, detergents interacting with biological membranes can result in the formation of detergent-lipid-protein micelles, detergent-protein, detergent-lipid and lipid micelles [Jones *et al.*, 1987]. The extent of solubilization will depend on the nature of the protein, the detergent used and the detergent to protein ratio [Helenius & Simons, 1975]. Increasing the detergent to protein ratio will tend to favor the formation of detergent-protein micelles at the expense of losing enzymatic activity. As a result of a low detergent to protein ratio, membrane proteins will tend to aggregate and the micelles formed will vary in homogeneity with respect to 6- and 3'-activities. This may explain the equal distribution of the 6- and 3'-IFPT activities following ion exchange chromatography on Mono Q (Fig. 22), as reflected by the similar reaction product ratios obtained for the three peak fractions (Table 11) from peaks I, II and III (Fig. 22). Alternatively, the

separation of two (or several) membrane proteins, on an ion exchanger, may be hindered by the presence of "boundary" or "annulus" lipids. These lipids remain bound to membrane proteins following solubilization with detergents, even under optimal conditions, and seemingly stabilize or maintain the protein's native conformation and therefore its activity [Sandermann Jr., 1987].

In order to isolate the detergent-protein micelles and improve the resolution of both activities on an ion exchanger, gel filtration was performed on Sephacryl S-400 (Fig. 19). However, due to the instability of the eluted activity, further chromatography could not be carried out.

It is interesting to note that SDS-PAGE of the different activity peaks (Fig. 24) resulted in similar protein profiles with a common protein band migrating at approximately 31-32 kD. This result as well as other evidence (J. Saleeba, personal communication) strongly suggest that the 31-32 kD protein is the IFPT. It is not unexpected that the different IFPTs involved in isoflavone prenylation would have similar molecular weights as has previously been shown with other flavonoid conjugation enzymes [Ibrahim et al., 1987; Varin, 1992]. Confirmation of the identity of this protein will have to await further purification, internal microsequencing and the cloning of its gene.

G. PERSPECTIVES FOR FUTURE WORK

Having established that the prenylation of isoflavones in *L. albus* is catalyzed by distinct, position-specific membrane associated enzymes, future work can focus on:

1. Attempts to purify one or several of the isoflavone prenyltransferases to near homogeneity allowing for further characterization of the different proteins and
2. To obtain an amino acid sequence in order
3. To clone a PT gene to study the developmental regulation of metabolite synthesis
4. Transform a heterologous plant in order to constitutively express prenyltransferase activity, thus rendering it more resistant to fungal or microbial attack, and therefore, decreasing the need for the use of pesticides.

Furthermore, raising antibodies against these prenyltransferases will allow to study their compartmentation within the cell, and serological relationship with other IFPTs.

H. REFERENCES

- Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S-I., Itoh, N., Shibuya, M. and Fukami, Y. (1937) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 262: 5592-5595.
- Alonso, W. R. and Croteau, R. (1993) Prenyltransferases and cyclases. in: *Methods in Plant Biochemistry Vol. 9. Enzymes of Secondary Metabolism.* (P.J.Lea, ed.) Academic Press, London. pp. 239-260.
- Anderson, M. S., Muehlbacher, M., Street, I. P., Profitt, J and Dale Poulter, C.(1989) Isopentenyl diphosphate:dimethylallyl diphosphate isomerase-- an improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 264: 19169-19175.
- Barnard, G.F. and Popják, G. (1981) Human liver prenyltransferase and its characterization. *Biochim. Biophys. Acta* 661: 87-99.
- Beerhues, L. and Wiermann, R. (1988) Chalcone synthetases from spinach (*Spinacia oleracea* L.). II. Immunofluorescence and immunogold localization. *Planta* 173: 544-553.
- Biggs, D.R. (1975) Post-infectional compounds from the French bean *Phaseolus vulgaris*; Isolation and identification of genistein and 2',4',5,7-tetrahydroxyisoflavone. *Aust. J. Chem.* 28: 1389-1392.
- Biggs, D. R., Welle, R., Visser, F. R. and Grisebach, H. (1987) Dimethylallylpyrophosphate:3,9-dihydroxypterocarpan 10-dimethylallyl transferase from *Phaseolus vulgaris*-- identification of the reaction product and properties of the enzyme. *FEBS Lett.* 220: 223-226.
- Biggs, D.R., Welle, R. and Grisebach, H. (1990) Intracellular localization of prenyltransferases of isoflavonoid phytoalexin biosynthesis in bean and soybean. *Planta* 181: 244-248.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Burden, R.S. and Bailey, J.A. (1975) Structure of the phytoalexin from soybean. *Phytochemistry* 14: 1389-1390.

- Burden, R.S., Bailey, J.A. and Dawson, G.W. (1972) Structures of three new isoflavonoids from *Phaseolus vulgaris* infected with tobacco necrosis virus. *Tetrahedron Lett.* 41: 4175-4178.
- Chmiel, E., Suffeld, R. and Wiermann, R. (1983) Conversion of phloroglucinol-type chalcones by purified chalcone isomerase from tulip anthers and from *Cosmos* petals. *Biochem. Physiol. Pflanzen* 178: 139-146.
- Cho, M-J. and Harper, J.E. (1991) Effect of localized nitrate application on isoflavonoid concentration and nodulation in split-root systems of wild-type and nodulation-mutant soybean plants. *Plant Physiol.* 95: 1106-1112.
- Clastre, M., Batignies, B., Feron, G., Soler, E. and Ambid, C. (1993) Purification and characterization of geranyl diphosphate synthase from *Vitis vinifera* L. cv Muscat de Frontignan cell cultures. *Plant Physiol.* 102: 205-211.
- Criado, M., Eibl, H. and Barrantes, F.J. (1984) Functional properties in model lipid membranes. Differential effects of chain length and head group of phospholipids on receptor affinity states and receptor-mediated ion translocation. *J. Biol. Chem.* 259: 9188-9198.
- Croteau, R. and Purkett, P.T. (1989) Geranyl pyrophosphate synthase: characterization of the enzyme and evidence that this chain-length specific prenyltransferase is associated with monoterpene biosynthesis in sage (*Salvia officinalis*). *Arch. Biochem. Biophys.* 271: 524-535.
- Cruikshank, I.A.M. and Perrin, D.R. (1971) Studies on phytoalexins: XI. The induction, antimicrobial spectrum and chemical assay of phaseollin. *Phytopath. Z.* 70: 209-229.
- Cruikshank, I.A.M. and Perrin, D.R. (1960) Isolation of a phytoalexin from *Pisum sativum* L. *Nature* 187: 799-800.
- Cruikshank, I.A.M., Biggs, D.R. and Perrin, D.R. (1974) Phaseollin and phaseollidin relationships in infection-droplets on endocarp of *Phaseolus vulgaris*. *Physiol. Plant Pathol.* 4: 261-276.
- Dakora, F.D., Joseph, C.M. and Phillips, D.A. (1993) Alfalfa (*Medicago sativa* L.) root exudates contain isoflavonoids in the presence of *Rhizobium meliloti*. *Plant Physiol.* 101: 819-864.
- Darvill, A. G. and Albersheim, P. (1984) Phytoalexins and their elicitors--a defense against microbial infection in plants. *Ann. Rev. Plant Physiol.* 35: 243-275.

- Davisson, V.J., Woodside, A. B. and Poulter, C.D. (1985) Synthesis of allylic and homoallylic isoprenoid pyrophosphates. *Meth. Enzymol.* 110: 130-144.
- de la Fuente, M. Perez, L. M., Hashagen, U., Chayet, L., Rojas, C., Portilla, G. and Cori, O. (1981) Prenyltransferases from the flavedo of *Citrus sinensis*. *Phytochemistry* 20: 1551-1557.
- De Luca, V. and Ibrahim, R.K. (1985) Enzymatic synthesis of polymethylated flavonols in *Chrysosplenium americanum*. I. Partial purification and some properties of S-adenosyl-L-methionine:flavonol 3-, 6-, 7-, and 4'-O-methyltransferases. *Arch. Biochem. Biophys.* 238: 596-605.
- Dewick, P.M. (1988) Isoflavonoids. In: *The Flavonoids: Advances in Research Since 1980.* (J.B. Harborne, ed.) Chapman and Hall, New York., pp. 125-209.
- Dhillon, D.S. and Brown, S.A. (1976) Localization, purification, and characterization of dimethylallylpyrophosphate:umbelliferone dimethylallyltransferase from *Ruta graveolens*. *Arch. Biochem. Biophys.* 177: 74-83.
- Dixon, R.A. (1986) The phytoalexin response: eliciting, signaling and control of host gene expression. *Biol. Rev.* 61: 239-291.
- Dixon, R.A., Blyden, E.R., Robbins, M.P., Van Tunen, A.J. and Mol, J.N. (1988) Comparative biochemistry of chalcone isomerases. *Phytochemistry* 27: 2801-2808.
- Dixon, R.A., Dey, P.M. and Lamb, C.J. (1983) Phytoalexins: enzymology and molecular biology. Vol. 55. In: *Advances in Enzymology and Related Areas of Molecular Biology.* (A. Meister, ed.) John Wiley & Sons, New York. pp. 1-136.
- Dixon, R. A., Dey, P. M. and Whitehead, I. M. (1982) Purification and properties of chalcone isomerase from cell suspension cultures of *Phaseolus vulgaris*. *Biochim. Biophys. Acta* 715: 25-33.
- Djordjevic, M.A., Redmond, J.W., Batley, M. and Rolfe, B.G. (1987) Clovers secrete specific phenolic compounds which either stimulate or repress nod gene expression in *Rhizobium trifolii*. *EMBO J.* 6: 1173-1179.
- Dogbo, O. and Camara, B. (1987) Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from *Capsicum* chromoplasts by affinity chromatography. *Biochim. Biophys. Acta* 920: 140-148.

- Dudley, M. W., Green, T. R. and West, C. A. (1986) Biosynthesis of the macrocyclic diterpene casbene in castor bean (*Ricinus communis* L.) seedlings: the purification and properties of farnesyltransferase from elicited seedlings. *Plant Physiol.* 81: 343-348.
- Ebel, J. and Grisebach, H. (1984) Defense strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: a molecular analysis. *TIBS* 13: 23-27.
- Eberhardt, N. L. and Rilling, H. C. (1975) Prenyltransferase from *Saccharomyces cerevisiae*. Purification to homogeneity and molecular properties. *J. Biol. Chem.* 250: 863-870.
- Ellis, B. E. and Brown, S. A. (1974) Isolation of dimethylallyl pyrophosphate:umbelliferone dimethylallyltransferase from *Ruta graveolens*. *Can. J. Biochem.* 52: 734-738.
- Endo, T. and Suga, T. (1992) Demonstration of geranyl diphosphate synthase in several higher plants. *Phytochemistry* 31: 2273-2275.
- Ferrer, M. A., Pedreño, M. A., Muñoz, R. and Ros Barceló, A. (1992) Constitutive isoflavones as modulators of indole-3-acetic acid oxidase activity of acidic cell wall isoperoxidases from lupin hypocotyls. *Phytochemistry* 31: 3681-3684.
- Firmin, J.L., Wilson, K.E., Rossen, L. and Johnston, A.W.B. (1986) Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. *Nature* 324: 90-92.
- Fisher, and Long, S.R. (1992) *Rhizobium*-plant signal exchange. *Nature* 357: 655-660.
- Fotsis, T., Pepper, M., Adlercreutz, H., Fleischmann, G., Hase, T., Montesano, R. and Schweigerer, L. (1993) Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. *Proc. Natl. Acad. Sci. (USA)* 90: 2690-2694.
- Fukui, H., Egawa, H., Koshimizu, K. and Mitsui, T. (1973) A new isoflavone with antifungal activity from immature fruits of *Lupinus luteus*. *Agr. Biol. Chem.* 37: 417-421.
- Gagnon, H. (1993) The isoflavonoids of *Lupinus albus*, their constitutive and environmentally induced accumulation and secretion, and their effect on bacterial growth. M. Sc. Thesis. Concordia University, Montréal.

- Gagnon, H., Séguin, J., Bleichert, E., Tahara, S. and Ibrahim, R. K. (1992) Biosynthesis of white lupin isoflavonoids from [U-¹⁴C]L-phenylalanine and their release into the culture medium. *Plant Physiol.* 100: 76-79.
- Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.
- Gershenzon, J. and Croteau, R. (1990) Regulation of monoterpene biosynthesis in higher plants. *Rec. Adv. Phytochem.* 24: 99-160.
- Green, T. R., Dennis, D. T. and West, C. A. (1975) Compartmentation of isopentenyl pyrophosphate isomerase and prenyltransferase in developing castor bean endosperm. *Biochem. Biophys. Res. Commun.* 64: 976-981.
- Green, T.R. and West, C.A. (1974) Purification and characterization of two forms of geranyl transferase from *Ricinus communis*. *Biochemistry* 13: 4720-4729.
- Hagmann, M. and Grisebach, H. (1984) Enzymatic rearrangement of flavanone to isoflavone. *FEBS Lett.* 175: 199-202.
- Hallard, D., Bleichert, E., Gagnon, H., Tahara, S. and Ibrahim, R. (1992) Production and release of isoflavonoids by lupin cell cultures. *Z. Naturforsch.* 47c: 346-352.
- Hamerski, D., Schmitt, D. and Matern, U. (1990) Induction of two prenyltransferases for the accumulation of coumarin phytoalexins in elicitor-treated *Ammi majus* cell suspension cultures. *Phytochemistry* 29: 1131-1135.
- Hanley, K. and Chappell, J. (1992) Solubilization, partial purification, and immunodetection of squalene synthetase from tobacco cell suspension cultures. *Plant Physiol.* 98: 215-220.
- Harborne, J.B. and Ingham, J.L. (1978) Biochemical aspects of the coevolution of higher plants with their fungal parasites. in: *Biochemical Aspects of Plant and Animal Coevolution.* (J.B. Harborne, ed.) Academic Press, London. pp. 343-405.
- Harborne, J. B., Ingham, J. L., King, L. and Payne, M. (1976) The isopentenyl isoflavone luteone as a pre-infectious antifungal agent in the genus *Lupinus*. *Phytochemistry* 15: 1485-1487.

- Heide, L. and Berger, U. (1989) Partial purification and properties of geranylpyrophosphate synthase from *Lithospermum erythrorhizon* cell cultures. *Arch. Biochem. Biophys.* 273: 331-338.
- Heide, L. and Tabata, M.. (1987) Geranylpyrophosphate:p-hydroxybenzoate geranyltransferase activity in extracts of *Lithospermum erythrorhizon* cell cultures. *Phytochemistry* 26: 1651-1655.
- Helenius, A. and Simons, K. (1975) Solubilization of membranes by detergents. *Biochim. Biophys. Acta* 415: 29-79.
- Heller, W. and Forkmann, G. (1988) Biosynthesis. In: *The Flavonoids: Advances in Research Since 1980.* (J.B. Harborne, ed.) Chapman and Hall, New York., pp: 399-425.
- Hinderer, W., Flentje, U. and Barz, W. (1987) Microsomal isoflavone 2'- and 3'-hydroxylases from chickpea (*Cicer arietinum* L.) cell suspensions induced for pterocarpan phytoalexin formation. *FEBS Lett.* 214: 101-106.
- Hirsh, A.M. (1992) Developmental biology of legume nodulation. *New Phytol.* 122: 211-237.
- Homans, A. and Fuchs, A. (1970) Direct bioautography on thin-layer chromatography as a method for detecting fungitoxic substances. *J. Chromatog.* 51: 327-329.
- Hrazdina, G. Zobel, A. and Hoch, H. C. (1987) Biochemical, immunological and immunocytochemical evidence for the association of chalcone synthase with endoplasmic reticulum membranes. *Proc. Natl. Acad. Sci. (USA)* 84: 8966-8970.
- Hungria, M., Joseph, C.M. and Phillips, D.A. (1991) *Rhizobium nod* gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). *Plant Physiol.* 97: 759-764.
- Ibrahim, R.K., De Luca, V., Khouri, H.E., Latchinian, L., Brisson, L. and Charest, P.M. (1987) Enzymology and compartmentation of polymethylated flavonol glucosides in *Chrysozplenium americanum*. *Phytochemistry* 26: 1237-1245.
- Ibrahim, R. K. and Varin, L. (1993) Flavonoid Enzymology. In: *Methods in Plant Biochemistry Vol. 9. Enzymes of Secondary Metabolism.* (P. J. Lea, ed.) Academic Press, London. pp. 99-131.
- Ingham, J.L. (1976) Induced isoflavonoids from fungus-infected stems of pigeon pea (*Cajanus cajan*). *Z. Naturforsch.* 31c: 504-508.

- Ingham, J.L. (1983) Naturally occurring isoflavonoids (1855-1981). In: Progress in the Chemistry of Organic natural Products, Vol. 43. (W. Herz, H. Grisebach, & G.W. Kirby, eds.) Springer-Verlag, New York. pp. 1-266.
- Ingham, J. L., Keen, N. T. and Hymowitz, T. (1977) A new isoflavone phytoalexin from fungus-inoculated stems of *Glycine wightii*. *Phytochemistry* 16: 1943-1946.
- Ingham, J. L., Tahara, S. and Harborne, J. B. (1983) Fungitoxic isoflavones from *Lupinus albus* and other *Lupinus* species. *Z. Naturforsch.* 38c: 194-200.
- Inglese, J., Koch, W. J., Caron, M. G. and Lefkowitz, R. J. (1992) Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature* 359: 147-150.
- Jansen, C. (1990) Biochromatography: separation of biopolymers in biotechnology. *GIT Fachzeitschrift für das Laboratorium* 34: 141-145. (Technical Bulletin from E. Merck, Darmstadt).
- Jones, O.T., Earnest, J.P. and McNamee, M.G. (1987) Solubilization and reconstitution of membrane proteins. In: Biological Membranes: A Practical Approach. Oxford University Press, Oxford. 1987. (Findlay, J.B.C. and Evans, W.H., eds.) pp. 139-177.
- Khouri, H.E. and Ibrahim, R.K. (1987) Resolution of five position-specific flavonoid O-methyltransferases by fast protein liquid chromatofocusing. *J. Chromatogr.* 407: 291-297.
- Khouri, H.E., Tahara, S. and Ibrahim, R.K. (1988a) Partial purification, characterization, and kinetic analysis of isoflavone-5-O-methyltransferase from yellow lupin roots. *Arch. Biochem. Biophys.* 262: 592-598.
- Khouri, H.E., De Luca, V. and Ibrahim, R.K. (1988b) Enzymatic synthesis of polymethylated flavonols in *Chrysosplenium americanum*. III. Purification and kinetic analysis of S-adenosyl:L-methionine:3-methylquercetin 7-O-methyltransferase. *Arch. Biochem. Biophys.* 265: 1-7.
- Klarman, W.L. and Sanford, J.B. (1968) Isolation and purification of an antifungal principle from infected soybeans. *Life Sci.* 7 (Part II): 1095-1103.
- Kleinig, H. (1989) The role of plastids in isoprenoid biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40: 39-59.

- Kochs, G. and Grisebach, H. (1986) Enzymatic synthesis of isoflavones. *Eur. J. Biochem.* 155: 311-318.
- Kosslak, R.M., Bookland, R., Barkei, J., Paaren, H.E. and Applebaum, E.R. (1987) Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max.* *Proc. Natl. Acad. Sci. (USA)* 84: 7428-7432.
- Koyama, T., Obata, S., Osabe, M., Takeshita, A., Yokoyama, K., Uchida, M., Nishino, T. and Ogura, K. (1993) Thermostable farnesyl diphosphate synthase of *Bacillus stearothermophilus*: molecular cloning, sequence determination, overproduction, and purification. *J. Biochem.* 113: 355-363.
- Kreuzaler, F. and Hahlbrock, K. (1975) Enzymic synthesis of an aromatic ring from acetate units. Enzymic purification and some properties of flavanone synthase from cell-suspension cultures of *Petroselinum hortense*. *Eur. J. Biochem.* 56: 205-213.
- Krishnan, H.B. and Pueppke, S.G. (1993) Flavonoid inducers of nodulation genes stimulate *Rhizobium fredii* USDA257 to export proteins into the environment. *Mol. Plant-Microbe Interactions* 6: 107-113.
- Kuc, J. and Rush, J. S. (1985) Phytoalexins. *Arch. Biochem. Biophys.* 236: 455-472.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- Lamb, C.J., Lawton, M.A., Dron, M. and Dixon, R.A. (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell* 56: 215-224.
- Lane, G. A., Biggs, D. R., Russell, G. B., Sutherland, O. R. W., Maree Williams, E., Maindonald, J. H. and Donnell, D. J. (1985) Isoflavonoid feeding deterrents for *Costelytra zealandica*: structure-activity relationships. *J. Chem. Ecol.* 11: 1713-1735.
- Latchinian, L., Khouri, H.E. and Ibrahim, R.K. (1987) Fast protein affinity chromatography of two flavonoid glucosyl-transferases. *J. Chromatogr.* 388: 235-242.
- Long, S.R. (1989) *Rhizobium*-legume nodulation: life together in the underground. *Cell* 56: 203-214.

- Lyne, R.L., Mulheirn, L.J. and Leworthy, D.P. (1976) New pterocarpinoid phytoalexins of soybean. *J. Chem. Soc., Chem. Commun.* 13: 497-498.
- Lyne, R.L. and Mulheirn, L.J. (1978) Minor pterocarpinoids of soybean. *Tetrahedron Lett.* 34: 3127-3128.
- Marshall, C. J. (1993) Protein prenylation: a mediator of protein-protein interactions. *Science* 259: 1865-1866.
- Miksicek, R.J. (1983) Commonly occurring plant flavonoids have estrogenic activity. *Molecular Pharmacol.* 44: 37-43.
- Miriyama, M., Tahara, S., Ingham, J. L. and Mizutani, J. (1992) Isoflavones from the root bark of *Piscidia erythrina*. *Phytochemistry* 31: 683-687.
- Monsavi, Y. and Adlercreutz, H. (1993) Genistein is an effective stimulator of sex hormone-binding globulin production in hepatocarcinoma human liver cancer cells and suppresses proliferation of these cells in culture. *Steroids* 58: 301-304.
- Nap, J-P. and Bisseling, T. (1990) Developmental biology of plant-prokaryote symbiosis: the legume root nodule. *Science* 250: 948-954.
- Ogura, K., Nishino, T., Shinka, T. and Seto, S. (1985) Prenyltransferases of pumpkin fruit. *Meth. Enzymol.* 110: 167-171.
- Ogura, K., Shinka, T. and Seto, S. (1972) The purification and properties of geranylgeranyl pyrophosphate synthase from pumpkin fruit. *J. Biochem.* 72: 1101-1108.
- Partridge, J.E. and Keen, N.T. (1976) Association of the phytoalexin kievitone with single-gene resistance of cow peas to *Phytophthora vignae*. *Phytopathology* 66: 426-429.
- Perez, L. M., Lozada, R. and Cori, O. (1983) Biosynthesis of allylic isoprenoid pyrophosphates by an enzyme preparation from the flavedo of *Citrus paradisi*. *Phytochemistry* 22: 431-433.
- Peterson, G. and Barnes, S. (1991) Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. *Biochim. Biophys. Res. Commun.* 179: 661-667.

- Phillips, D. A. (1992) Flavonoids: plant signals to soil microbes. *Rec. Adv. Phytochem.* 26: 210-231.
- Poulter, C. D. and Rilling, H. C. (1981) Prenyltransferases and Isomerase. In: *Biosynthesis of Isoprenoid Compounds, Vol. 1.* (John W. Porter and Sandra L. Spurgeon, eds.) John Wiley and Sons, New York. pp: 162-224.
- Sadowsky, M.J., Olson, E.R., Foster, V.E., Kosslak, R.M. and Verma, D.P.S. (1988) Two host-inducible genes of *Rhizobium fredii* and characterization of the inducing compound. *J. Bacteriol.* 170: 171-178.
- Sandermann, Jr., H. (1978) Regulation of membrane enzymes by lipids. *Biochim. Biophys. Acta* 515: 209-237.
- Schmelzer, E., Jahnen, W. and Hahlbrock, K. (1988) *In situ* localization of light-induced chalcone synthase mRNA, chalcone synthase, and flavonoid end products in epidermal cells of parsley leaves. *Proc. Natl. Acad. Sci. (USA)* 85: 2989-2993.
- Schröder, G., Zähringer, U., Heller, W., Ebel, J. and Grisebach, H. (1979) Biosynthesis of antifungal isoflavonoids in *Lupinus albus*. Enzymatic prenylation of genistein and 2'-hydroxygenistein. *Arch. Biochem. Biophys.* 194: 635-636.
- Shutt, D. A. (1976) The effects of plant oestrogens on animal reproduction. *Endeavour* 35: 110-113.
- Smith, D. A. and Banks, S. W. (1986) Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. *Phytochemistry* 25: 979-995.
- Smith, D.A., van Etten, H.D. and Bateman, D.F. (1973) Kievitone: the principal antifungal component of "substance II" isolated from *Rhizoctoma*-infected bean tissues. *Physiol. Plant Pathol.* 3: 179-186.
- Stafford, H. A. (1990) *Flavonoid Metabolism.* CRC Press, Inc. Boca Raton, FL. pp: 133-158.
- Steward, F.C., Caplin, S.M. and Millar, F.K. (1952) Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. *Ann. Bot.* 16: 57-78.
- Suga, T. and Endo, T. (1991) Geranyl diphosphate synthase in leaves of *Pelargonium roseum*. *Phytochemistry* 30: 1757-1761.

- Tahara, S. and Ibrahim, R.K. (1994). Prenylated isoflavonoids- an update. *Phytochemistry* (in press)
- Tahara, S., Ingham, J. L., Nakahara, S., Mizutani, J. and Harborne, J. B. (1984) Fungitoxic dihydrofuranoisoflavones and related compounds in white lupin, *Lupinus albus*. *Phytochemistry* 23: 185-190.
- Tahara, S., Katagiri, Y., Ingham, J.L. and Mizutani, J. (1994) Prenylated flavonoids in the roots of yellow lupin. *Phytochemistry* 36: 1261-1271.
- Tahara, S. Orihara, S., Ingham, J. L. and Mizutani, J. (1989) Seventeen isoflavonoids from *Lupinus albus* roots. *Phytochemistry* 28: 901-911.
- Tanford, C. and Reynolds, J.A. (1976) Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta* 457: 133-170.
- UC Berkeley Wellness Letter, October 1993.
- Varin, L. (1992) Flavonoid sulfation: phytochemistry, enzymology and molecular biology. *Rec. Adv. Phytochem.* 26: 233-254.
- Weinstein, L.I., Hahn, M.J. and Albersheim, P. (1981) Host-pathogen interactions. XVIII. Isolation and biological activity of glycinol, a pterocarpan phytoalexin synthesized by soybeans. *Plant Physiol.* 68: 358-363.
- Welle, R. and Grisebach, H. (1991) Properties and solubilization of the prenyltransferase of isoflavonoid phytoalexin biosynthesis in soybean. *Phytochemistry* 30: 479-484.
- Yanagihira, K., Ito, A., Toge, T. and Numoto, M. (1993) Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer* 53: 5815-5821.
- Yang, Z., Cramer, C. L. and Watson, J. C. (1993) Protein farnesyltransferase in plants--molecular cloning and expression of a homolog of the β subunit from the garden pea. *Plant Physiol.* 101: 667-674.
- Zähringer, U., Ebel, J., Mulheirn, L. J., Lyne, R. L. and Grisebach, H. (1979) Induction of phytoalexin synthesis in soybean--dimethylallyl pyrophosphate:trihydroxypterocarpan dimethylallyl transferase from elicitor-induced cotyledons. *FEBS Lett.* 101: 90-92.

- Zähringer, U., Schaller, E. and Grisebach, H. (1981) Induction of phytoalexin synthesis in soybean. Structure and reactions of naturally occurring and enzymatically prepared prenylated pterocarpans from elicitor-treated cotyledons and cell cultures of soybean. *Z. Naturforsch.* 36c: 234-241.
- Zhang, D., Jennings, S.M., Robinson, G.W. and Poulter, C.D. (1993) Yeast squalene synthase: expression, purification, and characterization of soluble recombinant enzyme. *Arch. Biochem. Biophys.* 304: 133-143.