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EPIGENETIC CONTROL OF A CELL WALL SCOPOLETIN PEROXIDASE BY LUPISOFLAVONE IN *LUPINUS*

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Key Word Index—*Lupinus albus*; Leguminosae; lupin; conformational isomerization; cell wall peroxidases; isoflavones; lupisoflavone.

Abstract—Lupisoflavone induces the conversion of both the C_1 and C_2 cell wall isoperoxidases to the C_5 isoperoxidase which possesses scopoletin-peroxidase activity. No other isoflavones isolated from lupin hypocotyls induce this conformational change of these cell wall enzymes.

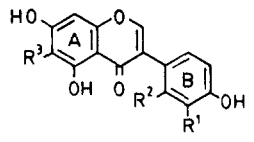
INTRODUCTION

In the course of our investigation into the nature of the enzymatic polymorphism of cell wall isoperoxidases, we have shown that a cell wall C_5 isoperoxidase, which shows a broad band on gel isoelectrofocusing, and which possesses scopoletin peroxidase activity, can be converted into two isoperoxidases, named C_1 and C_2, without apparent scopoletin peroxidase activity, after removal of phenolic compounds on Sephadex G-25 [1]. The first attempts to characterize this conformational equilibrium were greatly hindered by the instability of the conformers when standard techniques of protein purification were used [1].

In this report, we show that the joint conversion of the C_1 and C_2 isoperoxidases to the C_5 scopoletin peroxidase can be improved by incubation at neutral pH of an IEF-purified cell wall fraction containing the C_1 and C_2 isoperoxidases with lupisoflavone [5,7,4′-trihydroxy-3′-methoxy-6-(3,3-dimethylallyl)isoflavone (**1**)], a constitutive 5-hydroxylated isoflavone (aglycone) from lignifying lupin hypocotyls.

RESULTS AND DISCUSSION

Three major lupin isoflavones 2′-hydroxygenistein (**2**), wighteone (**3**), luteone (**4**), and minor amounts of genistein (**5**), were present in the EtOAc fractions isolated from lupin hypocotyls in the pre-exponential (5-day-old) and exponential (7-day-old) growth phases of development. However, during the post-exponential growth phase (15-day-old), the appearance of a new faint Gibbs-positive spot at R_f 0.58 on the developed TLC plates ($CHCl_3$–MeOH, 20:1) was observed. This new spot was identified as lupisoflavone (**1**) on the basis of its R_f in several solvent systems, its colour with Gibbs reagent, its UV spectroscopic characteristics, the shifts induced in its UV (methanol) spectrum after addition of NaOH, $AlCl_3$, NaOAc, and H_3BO_3 (see Experimental), and by comparison of this data with literature data [2].



	R^1	R^2	R^3
1	OMe	H	$CH_2CH{=}CMe_2$
2	H	OH	H
3	H	H	$CH_2CH{=}CMe_2$
4	H	OH	$CH_2CH{=}CMe_2$
5	H	H	H

The presence of lupisoflavone in the hypocotyls during the post-exponential growth phase coincided temporally with the expression of a cell wall peroxidase which is able to oxidize scopoletin and which has been considered before as an enzyme marker of xylogenesis in lupin [3]. This scopoletin peroxidase can be converted to two isoperoxidases, named C_1 and C_2, after removal of phenols of the cell wall protein preparation by chromatography on Sephadex G-25 [1]. The possibility that lupisoflavone can control this conformational equilibrium was further investigated.

When purified cell wall isoperoxidase fractions containing both the C_1 and C_2, but not the C_5 isoperoxidase, were incubated with lupisoflavone, and subsequently separated by isoelectric focusing, the appearance of the C_5 scopoletin peroxidase was observed in the zymographic patterns obtained by staining with benzidine (Table 1). This effect was reversed by filtration on Sephadex G-25 of the C_1 and C_2 isoenzyme fraction treated with lupisoflavone (Table 1), and was dependent on the incubation pH, since at acidic pH (5.0) this interconversion was not observed (Table 1).

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The interconversion of C_1 and C_2 to C_5 isoperoxidase mediated by isoflavones was specific for lupisoflavone, since incubation of the C_1 and C_2 isoenzyme fraction with luteone, wighteone, genistein, or 2'-hydroxygenistein, gave no scopoletin-peroxidase band on zymogrammes. Thus, the structural requirements of lupin isoflavones needed to induce the C_{1-2} to C_5 interconversion are stringent and require that the isoflavones possess a B ring of the guaiacyl type.

A characteristic of the cell wall C_{1-2} isoperoxidases is their high specificity towards guaiacyl-type phenols [1], and their inability to oxidize coumarins, such as scopoletin (7-hydroxy-6-methoxycoumarin) [1]. Since the C_{1-2} isoperoxidases are able to oxidize lupisoflavone in the presence of hydrogen peroxide and also scopoletin if the reaction media additionally contains lupisoflavone and hydrogen peroxide (Table 2), lupisoflavone could operate as an intermediate in the single hydrogen atom transfer from scopoletin to the oxy-heme moiety of cell wall isoperoxidases during the catalytic cycle, according to Scheme 1. Linkage of lupisoflavone to both C_1 and C_2 isoperoxidases could therefore be an epigenetic mechanism of modification of the catalytic properties of these cell wall isoenzymes, expressed differentially during lignification [3]. Its physiological role, though, remains unclear.

EXPERIMENTAL

Plant material. Hypocotyls of *Lupinus albus* L. cv. multolupa were grown in darkness as previously described [1].

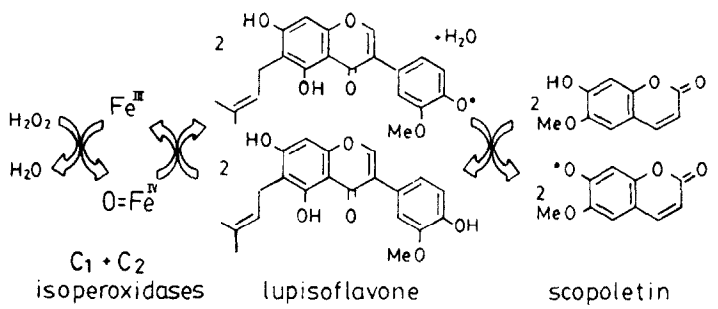
Table 1. Conversion of both the C_1 and C_2 to the C_5 isoperoxidase by lupisoflavone

		Cell wall isoperoxidase		
pH	Lupisoflavone	C_1 (nkat)	C_2 (nkat)	C_5 (nkat)
pH 7.5	—	0.43	0.46	0.0
pH 7.5	+	0.01	0.01	0.93
pH 7.5 + Sephadex G-25	+	0.45	0.46	0.0
pH 5.0	+	0.44	0.45	0.0

Table 2. Oxidation of scopoletin and lupisoflavone by both the C_1 and C_2 cell wall isoperoxidases in the presence of hydrogen peroxide

	Substrate oxidized	
Substrate added	Scopoletin (A_{460})	Lupisoflavone (spot at R_f 0.0)*
Lupisoflavone		+ + +
Scopoletin	0.00	
Lupisoflavone + scopoletin	0.16	—

*R_f value in both $CHCl_3$–MeOH (20:1) and $CHCl_3$–Me_2CO–aq. (35%) NH_3 (70:60:1).



Scheme 1. Proposed role of lupisoflavone as an intermediate in the single hydrogen atom transfer from scopoletin to the oxyheme moiety of both the C_1 and C_2 cell wall isoperoxidases.

Cell wall enzyme fraction. Cell wall-bound peroxidases were isolated from 15-day-old hypocotyls as reported [1]. The C_1 and C_2 isoperoxidases were purified by Sephadex G-25 filtration and gel isoelectric focusing (IEF) [1]. The gel was sliced into 2.5 mm sections, and both the C_1 and C_2 isoperoxidases were eluted overnight with 50 mM Tris–HCl, pH 7.2.

Isolation of isoflavones. Exponentially growing (7-day-old) and lignifying (15-day-old) hypocotyls (250 g) were cut into small pieces which were placed in a beaker and covered with MeOH. After standing for 24 hr at 4°, the suspension was ground in MeOH. The combined MeOH extracts were filtered by suction, and reduced to near dryness (*in vacuo*, 40°). The brown oily residue was defatted with hexane, partitioned (× 3) with EtOAc, and the combined EtOAc fractions were reduced *in vacuo* to 1 ml.

Aliquots (50 μg) were applied to silica gel TLC plates (Merck, F-254, layer thickness 0.5 mm), which were then developed in $CHCl_3$–MeOH (20:1) [4]. Detection of isoflavones on developed TLC plates was by inspection under long (365 nm) wavelength UV light, and by the characteristic colours formed with Gibbs reagent [2]. Spots exhibiting fluorescence at R_f 0.09 (2′-hydroxygenistein), 0.16 (genistein + luteone), 0.27 (wighteone) and 0.58 (lupisoflavone), were removed, eluted with EtOAc, and further purified by TLC (silica gel, 0.25 mm) in $CHCl_3$–Me_2CO–aq. (35%) NH_3 (70:60:1). This system separated genistein (R_f 0.11), found in small quantities, from luteone (R_f 0.22). Spots at R_f 0.48, 0.40, and 0.35, in $CHCl_3$–MeOH (20:1), corresponding to others Gibbs reagent-positive bands, were discarded since although these compounds were detectable on silica gel TLC plates, they were present in amounts insufficient to permit UV quantitation and characterization. All the above-mentioned lupin isoflavones ran as single bands when they were checked for homogenity by silica gel TLC in *n*-pentane–Et_2O–HOAc (75:25:6) [4].

Identification of isoflavones. Genistein [5,7,4′-trihydroxyisoflavone (**5**)], 2′-hydroxygenistein [5,7,2′,4′-tetrahydroxyisoflavone (**2**)], wighteone [5,7,4′-trihydroxy-6-(3,3-dimethylally)isoflavone (**3**)], and luteone [5,7,2′,4′-tetrahydroxy-6-(3,3-dimethylallyl)isoflavone (**4**)] were identified by their UV spectroscopic properties, colour reaction with Gibbs reagent [2], and R_f values [2].

Lupisoflavone [5,7,4′-*trihydroxy*-3′-*methoxy*-6-(3,3-*dimethylallyl*)*isoflavone* (**1**)]. Diazotised *p*-nitroaniline: orange; Gibbs reagent: blue; fluorescence: purple-black unaffected by NH_3 vapour under UV light (365 nm). UV λ_{max}^{MeOH} nm: 268, 292 sh; + NaOH, 281, 330 sh; + $AlCl_3$, 275, 327 sh; + NaOAc, 275, 342 (addition of solid H_3BO_3 regenerated the MeOH spectrum).

Incubation media. 0.5 ml of purified C_1 and C_2 cell wall isoperoxidases (1.0 nkat) were incubated with 10 μl (2 μg) of isoflavones in 0.1 M Tris–acetate buffered either at pH 5.0 or pH 7.5, for 20 min at 25°. At the end of the incubation period, cell wall isoperoxidases were separated by gel isoelectrofocusing [5], and enzymatic activities of the C_1, C_2, and C_5 isoperoxidases were determined quantitatively by measuring A_{460} of the isoperoxidase patterns obtained on using benzidine as an electron donor [5].

Oxidative media. 1 nkat of C_1 and C_2 cell wall isoperoxidases was incubated with 5 μg of lupisoflavone in the presence of 1 mM H_2O_2 in 0.5 ml of 50 mM Tris-HCl, pH 7.2, for 5 min at 25°. Controls were performed in the absence of enzyme. At the end of the incubation period, the reaction media were extracted with EtOAc (× 3), and the organic phase applied to silica gel TLC plates which were developed in $CHCl_3$–MeOH (20:1) or $CHCl_3$–Me_2CO–aq. (35%) NH_3 (70:60:1). The oxidation product(s) of lupisoflavone afforded, in both cases, a single spot of R_f 0.00, after spraying with diazotised *p*-nitroaniline reagent. Oxidation of scopoletin (2 mM) in the reaction media was followed by measurement of the increase in absorbance at 460 nm with respect to the control, for 5 min at 25°.

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