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THE ENZYMATIC SYNTHESIS OF AMYRIN GLUCOSIDE

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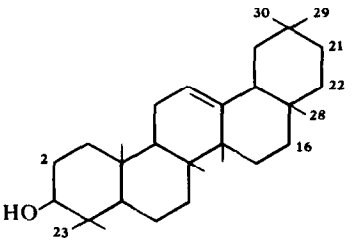
Key Word Index—*Pisum sativum*; Leguminosae; triterpene glucoside; UDP-glucose glucosyltransferase; membrane-bound enzymes; amyrin glucoside.

Abstract—Label appeared in several cell fractions isolated from the cotyledons of pea seeds germinated for 48 hr with mevalonate-[2-^{14}C]. The major radioactive metabolite in each fraction was amyrin. In a similar experiment, a fraction sedimenting between 1000 and 25000 *g* and a microsomal pellet were labeled with ^{3}H from mevalonate-[2-^{3}H]. Each of these tritiated fractions on incubation with UDP-glucose-[U-^{14}C] yielded $CHCl_3$–MeOH-soluble material bearing ^{14}C and ^{3}H. TLC of the extracts gave a compound chromatographically identical with a glucoside and bearing the two isotopes. Acid hydrolysis of this compound gave an ether-soluble material carrying ^{3}H alone. On TLC it co-chromatographed with amyrin. Of the two tritiated cotyledon fractions, the microsomal pellet had the lower glucosyltransferase activity. The labeled amyrin residing in this fraction served as an acceptor for glucose from UDP-glucose in the presence of a glucosyltransferase from pea seedling axis tissue. In such a mixed preparation, the axis tissue transferase suffers a marked inhibition by the cotyledon preparation.

INTRODUCTION

The triterpenoid saponins are a large group of compounds most commonly found and widely distributed in the plant kingdom [1, 2]. The vast majority of the triterpenoid sapogenins are β-amyrin (**1**) metabolites, many of which result from various combinations of oxidation of the C-23, C-28, C-29 and C-30 methyl groups and hydroxylation at the C-2, C-16, C-21 and C-22 positions. The synthesis of the 3β-D-glucuronoside of oleanolic acid (**1**, C-28 = —COOH) from oleanolic acid and UDP-glucuronic acid in cell-free preparations of *Calendula officinalis* seedlings has been reported [3]. β-Amyrin and erythrodiol (**1**, C-28 = —CH_2OH), the presumed biogenetic precursors of oleanolic acid, when added to such cell-free preparations did not undergo glucuronylation.



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Although β-amyrin is a major isoprenoid of pea seeds, we have been unable to demonstrate the glucosylation of exogenous β-amyrin by several different preparations with glucosylating activity from pea seedling tissue. In this paper we describe the *in vitro* glucosylation of the endogenous labeled amyrin of germinating pea seed cotyledons by a glucosyltransferase located in the cotyledon and also by that located in an axis tissue preparation.

RESULTS AND DISCUSSION

Doireau [4] has recently found α- and β-amyrin to occur in pea cotyledons. β-Amyrin represents approximately 80% of this triterpene fraction. As both compounds are structurally similar, in this work no attempt has been made to separate them and the fraction containing both compounds has been called the amyrin fraction.

The cotyledon has poor sterol glucosyltransferase activity as compared with axis tissue [5]. However, the potential glucose acceptors, the amyrin triterpenes, can be made radioactive almost exclusively from labeled mevalonate in such tissue, thereby providing an endogenous source of labeled amyrin to examine triterpene glucoside synthesis.

The distribution of ^{14}C in $CHCl_3$–MeOH extracts of several different centrifugation fractions of cotyledon homogenates obtained from peas germinated for 48 hr with MVA-[2-^{14}C] was determined. Extracts were analysed by TLC in ethyl acetate–hexane followed by ^{14}C scanning. In accord with a similar, previous study [6], most (>70%) of the radioactivity was associated with triterpene and <1% with sterol in the different fractions. The remainder appeared to be squalene or terpenoid esters. No labeled glucoside could be detected in any of the fractions when chromatographed in a $CHCl_3$–EtOH–H_2O solvent mixture [5]. Further examination of the triterpene fraction by Ag^+–SiO_2 TLC in benzene–hexane [7] showed cycloartenol and 24-methylene-cycloartanol to represent <5% of the triterpene. Thus, it is clear that in these cotyledon particulate preparations the labeled triterpene acceptor for glucosylation was essentially amyrin.

Two types of experiment were conducted: (i) Glucosylation of amyrin by a transferase located in the same particulate preparation. (ii) Glucosylation of amyrin in cotyledon preparations with a low transferase activity by an active glucosyltransferase preparation from pea seedling axis tissue.

Table 1. The ^3H and ^{14}C content of the isolated glycosides and free, unreacted amyrin fractions of the $CHCl_3$–MeOH extracts from reactions of fractions from pea seedling axis and ^3H-cotyledon tissue with UDP-glucose-[U-^{14}C]

Fraction	Glycoside (dpm)		Amyrin (dpm)
	^3H	^{14}C	^3H
(1) Cotyledon 25K	8800	1920	458000
(2) Cotyledon microsome	3400	400	319000
(3) Seedling axis tissue 25K	—	3150	
(4) (2) + (3)	5000	950	

The data are the average of duplicate reactions. Maximum deviation from the average was $<10\%$.
Details of the reactions are given in the Experimental.

^3H-Labeled fractions of cotyledon homogenates were prepared as before but using MVA-[2-^3H] as substrate. In addition, a sterol glucosyltransferase was prepared using 7-day-old pea seedling axis tissue [5]. After incubation of the appropriate fractions with UDP-glucose-[U-^{14}C] and Mg^{2+}, the isolation of radioactive lipid was made as previously described. The ^3H and ^{14}C content of the neutral glycoside and free, unreacted triterpene in the extracts from the reactions are shown in Table 1. Both cotyledon fractions produced a labeled glucoside carrying ^3H. This was shown to be amyrin by acid hydrolysis and TLC of the aglycone. Of the total labeled amyrin present, 1.9 and 1.1% of the 25K and microsomal fraction, respectively, was glucosylated. The ^{14}C content gives a measure of glucosyltransferase activity. The difference in the $^3H/^{14}C$ ratios between the two fractions is not meaningful because the specific activity of the amyrin in the two fractions was not known and the extent to which ^{14}C in the glucoside results from glucosylation of endogenous, unlabeled sterol was also unknown. However, it is notable that glucosyltransferase activity in the microsome fraction was low and thus offers an ideal preparation for glucosylation catalysed by another fraction. This is shown in experiment 4 in Table 1. It is clear that the transferase activity in this experiment was only approximately 1/4 of what might have been anticipated from the sum of the individual activities of fractions (2) and (3). The poorer than expected transferase activity may be a consequence of the presence in the microsome fraction of an active glucosidase. Kalinowska and Wojciechowski [8] have recently reported the presence of a β-glucosidase with high specificity for sterol β-glucosides in *Sinapis alba* seedlings. Alternatively, the cotyledon microsome fraction may contain inhibitors of the transferase or enzymes catalysing reactions competing for UDP-glucose, e.g. pyrophosphorylase. In spite of the diminished glucosyltransferse activity, there was nevertheless increased glucosylation of amyrin originating in the microsome fraction by the added axis tissue transferase.

From these experiments, it cannot be determined whether glucosylation occurred by the action of the axis tissue transferase on the amyrin while it was associated with the microsomal membrane, or if transfer of amyrin from the microsome to the axis tissue membrane was a prerequisite for glucosylation. For the latter to occur, there must presumably be a requirement for the amyrin to be associated initially with a membrane because all our attempts to bring about glucosylation with the axis glucosyltransferase using free, labeled triterpene dispersed by sonication have been unsuccessful.

The glucosylation of amyrin by a cell fraction known to have sterol glucosylating activity may reflect an acceptance of the triterpene as a substrate by the sterol glucosyltransferase. However, the presence of two methyl groups adjacent to the C-3 hydroxyl in amyrin would no doubt impose limitations on the binding of such a molecule to the transferase active site.

The aglycones of the β-amyrin triterpenoid saponins are invariably metabolites of β-amyrin [2]. The sequence in which glycosylation and modifications of the aglycone occur is unknown. The absence of amyrin itself as an aglycone of plant saponins may be a consequence of an extremely rapid modification of the β-amyrin moiety after glucosylation occurs at C-3 such that amyrin glucoside does not accumulate, or that glucosylation at C-3 before modification of the aglycone or its further glycosylation is only one, a very minor one, of several pathways to the final saponin. In this regard, Wojciechowski [3] has concluded from his studies on the synthesis of the oleanolic acid glycosides of *C. officinalis* that the formation of the glycosides takes place only after the oxidation step to convert β-amyrin to oleanolic acid.

In a previous communication [7], we speculated that saponins may be formed during the last stages of maturation of pea seeds. Such compounds, which are powerful surfactive agents, would be available for modifying the permeability of cotyledon membranes during the early stages of germination. The amyrin glucosylating activity located in the germinating pea cotyledon may represent the residual activity from the maturing seed. The amyrin conjugate described in this paper is presumed to be the glucoside though more work is necessary to demonstrate it definitively.

EXPERIMENTAL

Materials. Pea seeds, *Pisum sativum* L. cv Alaska, were from W. Atlee Burpee Co., Riverside, California. UDP-glucose-[U-^{14}C] (155 μCi/μmol) was obtained from International Chemical and Nuclear, Irvine, California. Unlabeled UDP-glucose was from Calbiochem, La Jolla, California. MVA-[2-^3H] (500 mCi/mmol) and MVA-[2-^{14}C] (40.8 mCi/mmol) were from New England Nuclear, Boston, Mass. TLC was carried out on plastic sheets coated with a 100 μm layer of Si gel and also with such sheets impregnated with $AgNO_3$ [7].

Methods. TLC solvent mixtures for the separation of the triterpenes and the glycosides and methods for their detection were the same as those previously described [5, 7]. TLC in $CHCl_3$–EtOH–H_2O (90:9:0.5) served to isolate the glycoside fraction. TLC in EtOAc–hexane (12:88) separated the triterpene fraction and TLC of the acetates of this fraction on $AgNO_3$-impregnated SiO_2 sheets in C_6H_6–hexane (2:3) separated cycloartenol, 24-methylenecycloartanol and amyrin. Standards

of cholesterol glucoside, β-amyrin and the 3 triterpene acetates were run where appropriate.

Radioactivity measurements. All extracts were evapd to dryness in vials followed by addition of 4 ml of scintillation fluor. Location of radioactive zones on TLC sheets was made by scanning with a Packard radiochromatogram scanner. The zones were scraped into vials and mixed with fluor for counting. Efficiency of counting was determined by spiking samples with either standard toluene-[^{14}C] or toluene-[^{3}H]. Counting of ^{3}H and ^{14}C in double-labeling experiments was determined by liquid scintillation spectroscopy.

Preparation of cotyledon glucosyltransferase carrying ^{3}H-*labeled triterpene.* 20 pea seeds were germinated with MVA-[2-^{3}H] (25 μCi) for 48 hr. The cotyledons (8.5 g) were excised and ground in an ice-cold mortar with 25 ml 50 mM Tris–HCl (pH 8). The slurry was squeezed through Miracloth and the filtrate centrifuged at 1000 *g* for 5 min. The resulting pellet was discarded and the supernatant recentrifuged at 25000 *g* for 20 min to give a pellet designated the 25K fraction. The supernatant from this was recentrifuged at 105000 *g* for 90 min to give a microsomal pellet. Both the 25K and microsomal pellets were resuspended in 50 mM Tris–HCl (pH 8) buffer.

Preparation of ^{14}C-*labeled fractions from cotyledons.* 20 pea seeds were germinated with MVA-[2-^{14}C] (2 μCi) and processed in a similar way to that described for the ^{3}H-labeled fractions. ^{14}C-Labeled fractions were obtained which sedimented at 1000 *g* in 5 min, 25000 *g* in 20 min and 105000 *g* in 90 min and also retained was the supernatant fraction.

^{14}C-*Distribution in centrifugal fractions from cotyledons.* One half of each fraction was made basic (pH 10) with dil. NaOH and then extracted 3× with equal vols. of $CHCl_3$–MeOH (2:1). A sample of each evapd extract was analysed for glycoside formation by TLC in $CHCl_3$–EtOH–H_2O (90:9:0.5). A second sample was run in an EtOAc–hexane (12:88) solvent mixture. The separated triterpene zone, located by the radiochromatogram scanner, was eluted with $CHCl_3$–MeOH (2:1). The evapd extracts were acetylated with Ac_2O–Py and the acetates subjected to TLC on $AgNO_3$-impregnated SiO_2 sheets in C_6H_6–hexane (2:3) in order to quantitate the distribution of ^{14}C among amyrin, cycloartenol and 24-methylene-cycloartanol.

Preparation of sterol: UDP-*glucose glucosyltransferase.* This was isolated from the axis tissue of 7-day-old pea seedlings according to a previously described procedure [6].

Incubation of fractions with UDP-*glucose*-[U-^{14}C]. The 25K and microsomal pellets from the ^{3}H-cotyledons and the glucosyltransferase from 7-day-old pea seedling axis tissue, suspended in 50 mM Tris–HCl (pH 8), were the sources of enzyme activity. All incubations contained 5 μmol Mg^{2+}, 2 μmol (2 μCi) UDP-glucose-[U-^{14}C] and the enzyme made up to a total vol. of 2.5 ml with the Tris buffer. Four experiments were run in duplicate in which the enzyme in each replicate was: (i) 1/4 of the 25K fraction, (ii) 1/4 of the microsomal fraction, (iii) pea seedling axis glucosyltransferase (1 ml) and (iv) 1/4 the microsomal fraction and pea seedling axis glucosyltransferase (1 ml). All reactions were run for 1 hr at 28° then terminated by the addition of dil. NaOH to make the mixture pH 10, followed by 3 extractions with equal vols. of $CHCl_3$–MeOH (2:1). The ^{3}H and ^{14}C content was determined in the isolated glycoside fraction after TLC and re-TLC in the $CHCl_3$–EtOH–H_2O solvent mixture. The radioactive zone at the solvent front of the first TLC was eluted and rechromatographed in EtOAc–hexane to determine the ^{3}H-content of the amyrin fraction.

Acid hydrolysis of isolated glycoside. The evapd sample obtained from elution of the glycoside after the second TLC was heated under reflux in 0.5% H_2SO_4 in EtOH–H_2O (19:1) for 2 hr. After neutralization with NaOH the free triterpene was extracted with hexane.

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