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EVIDENCE FOR THE FORMATION OF LINAMARIN AND LOTAUSTRALIN IN FLAX SEEDLINGS BY THE SAME GLUCOSYLTRANSFERASE*

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Abstract—The ratio of glucosides formed from acetone cyanohydrin and butanone cyanohydrin by the UDP-glucose–ketone cyanohydrin glucosyltransferase from flax seedlings (*Linum usitatissimum* L.) remains constant during its purification. This suggests that the two cyanogenic glucosides, linamarin and lotaustralin, found in flax are produced *in vivo* by the same enzyme. This conclusion is supported by feeding experiments with precursors and intermediates of the two pathways. The rate of incorporation of radioactive valine or acetone cyanohydrin into linamarin is significantly reduced after preincubation of flax seedlings with isoleucine, the precursor amino acid of lotaustralin. Similarly, valine reduced the rate of incorporation of labeled isoleucine or butanone cyanohydrin into lotaustralin. It seems likely that the pool of the radioactive precursor in both pathways is diluted by unlabeled intermediates formed *in vivo* from either amino acid by the same enzymes.

INTRODUCTION

LINAMARIN (III) and lotaustralin (IV) are the two cyanogenic glucosides produced by the flax plant (*Linum usitatissimum* L.). With one exception, both of these glucosides are also present in all of those plants which so far have been reported to contain either linamarin or lotaustralin.[1–3] Moreover, genetic studies[4] on cyanogenesis in white clover (*Trifolium repens* L.) have shown that the presence or absence of the two glucosides is governed by the alleles of a single gene (Ac). These facts as well as the structural similarity of linamarin and lotaustralin suggest that these cyanogens are synthesized by the same enzymes.

In a previous communication we proposed a biosynthetic pathway for the formation of cyanogenic glucosides in higher plants.[5] More recently we have purified an enzyme UDP-glucose–ketone cyanohydrin β-glucosyltransferase from an acetone powder of flax seedlings which catalyzes the last step in the biosynthesis of linamarin and lotaustralin[6] (Fig. 1, I → III and II → IV). The enzyme which was purified about 120 fold exhibited a high degree of specificity for the aliphatic side chains of the substrate. Only cyanohydrins bearing methyl or ethyl groups were found to be readily glucosylated. The purification of this enzyme did

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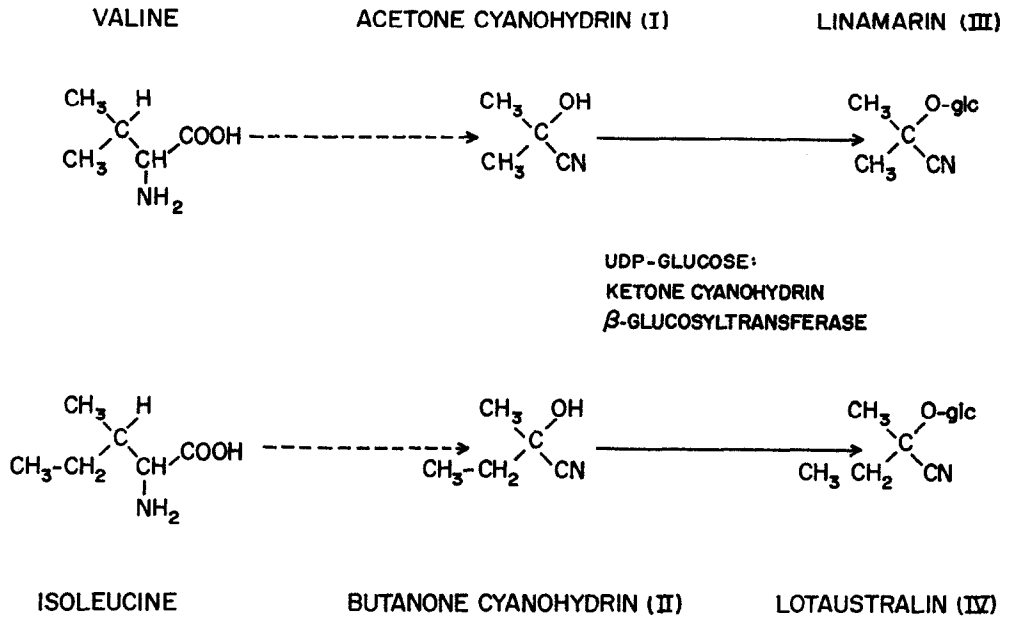

FIG. 1. SCHEME FOR THE BIOSYNTHESIS OF LINAMARIN AND LOTAUSTRALIN IN THE FLAX PLANT. The broken arrows indicate intermediates not listed in this scheme.

not result in a separation of two distinct enzyme fractions responsible for the formation of linamarin and lotaustralin, respectively. Results reported in this paper provide evidence that both cyanogenic glucosides in the flax plant are formed by the same enzyme.

RESULTS AND DISCUSSION

When the rate of glucosylation of acetone cyanohydrin (I) and butanone cyanohydrin (II) was followed through the purification procedure of the UDP-glucose–ketone cyanohydrin β-glucosyltransferase from flax seedlings,[6] the ratio of the two glucosides formed remained constant within the limits of experimental error (Table 1). Therefore it must be assumed that either the same enzyme catalyzes the formation of both glucosides or that two enzymes with very similar physical properties are present and were not separated by the methods employed in this purification procedure.

In order to obtain more information on this point, feeding experiments with flax seedlings were designed to show whether precursors of one cyanogenic glucoside might interfere with the biosynthesis of the other. If the conversion of valine and isoleucine to linamarin and lotaustralin, respectively (Fig. 1) is catalyzed by the same enzymes, the biosynthetic intermediates of one glucoside should compete *in vivo* with those of the other as natural substrates of these enzymes. Thus they would dilute out the pool of radioactive intermediates when labeled compounds are administered to the plant.

Results of such a competition experiment are listed in Table 2. After feeding 50 μmoles of non-radioactive valine (VAL) or isoleucine (ILE) to ten 5-day-old flax seedlings for 1 hr, 10 μmoles of the ^{14}C-labeled amino acid were administered to the plants for an incubation period of 5 hr. The cyanogenic glucosides were then extracted from the seedlings and

TABLE 1. RATIO OF GLUCOSIDES FORMED UNDER STANDARD ASSAY CONDITIONS FROM BUTANONE CYANOHYDRIN (BCH) AND ACETONE CYANOHYDRIN (ACH) BY PROTEIN FRACTIONS OBTAINED DURING THE PURIFICATION OF UDP-GLUCOSE–KETONE CYANOHYDRIN β-GLUCOSYLTRANSFERASE FROM FLAX SEEDLINGS

	Ratio of glucosylation (BCH:ACH)	
Purification step*	Expt. 1	Expt. 2
Crude extract	0·9	1·1
$MnCl_2$ fractionation	1·0	—
Precipitation with $(NH_4)_2SO_4$	1·1	—
Dowex-1 treatment	1·1	1·4
$(NH_4)_2SO_4$ fractionation steps		
0–35%	1·2	1·0
35–50%	1·1	1·4
50–80%	1·1	1·5
DEAE cellulose column	—	0·9
Sephadex G-100 column	—	1·3

* See Ref. 6 for description of purification procedure.

TABLE 2. FEEDING EXPERIMENTS WITH SHOOTS OF 5-day-old FLAX SEEDLINGS

Expt.	Amino acid used in pretreatment	^{14}C amino acid administered	Total radioactivity isolated (dis/min)	Distribution of radioactivity in amino acid and glucoside (%)	Labeled glucoside (per cent of control)
	None	VAL	103,300	VAL 83·3 LIN 16·7	100
1	VAL	VAL	86,300	VAL 96·4 LIN 3·6	22
	ILE	VAL	70,640	VAL 92·7 LIN 7·3	44
	None	ILE	118,600	ILE 93·2 LOT 6·8	100
2	VAL	ILE	69,500	ILE 98·3 LOT 1·7	25
	ILE	ILE	85,780	ILE 97·5 LOT 2·5	37

Radioactive valine (VAL) and isoleucine (ILE) were administered after the plants were pretreated with unlabeled amino acids. The tissue was then extracted, and the labeled cyanogenic glucosides, linamarin (LIN) and lotaustralin (LOT), were separated from their precursor amino acids valine and isoleucine, respectively. The percentage of radioactivity found in the glucosides is compared with the values obtained from control experiments in which the seedlings were not pretreated.

purified by paper chromatography. Since the uptake of radioactive label from the amino acid solution was rather low and somewhat variable (an average of about 30 per cent), the amounts of radioactivity isolated in the glucoside fractions could not be compared directly. Instead, the total radioactivity extractable in 80% ethanol from the plants after incubation was used as an internal standard, and the percentage of radioactivity in the labeled glucoside was then calculated. In previous experiments it has been shown by chromatography in several solvent systems that valine, isoleucine, linamarin and lotaustralin were not contaminated with other radioactive compounds in the solvent systems used.

In the first experiment (Table 2, Expt. 1) the relative incorporation of radioactive valine into linamarin was reduced to 44 per cent when the seedlings were preincubated with isoleucine, the precursor amino acid of lotaustralin. The same effect was observed with unlabeled valine itself probably due to dilution of the radioactive substrate. In a second experiment (Table 2, Expt. 2) the incorporation of labeled isoleucine into lotaustralin was similarly reduced when the seedlings were preincubated with valine.

Since the conversion of the appropriate precursor amino acid to linamarin or lotaustralin is affected equally by the addition of either amino acid, it seems likely that both pathways are catalyzed by the same enzymes. However, it can not be excluded that the transport of one of these amino acids within the plant is influenced by the other rather than its conversion to a cyanogenic glucoside. We therefore employed the more immediate precursors (Fig. 1), acetone cyanohydrin (ACH) and butanone cyanohydrin (BCH), as radioactive marker compounds to be converted to the corresponding glucosides. Again, both valine and isoleucine effectively reduced the rate of glucosylation of either cyanohydrin *in vivo* (Table 3). In this case, the uptake of radioactive substrates was over 99 per cent, and thus the values obtained for the amount of label found in the glucosides could be compared directly. These results can be explained by the assumption that the pool of radioactive cyanohydrin is diluted by unlabeled cyanohydrins formed enzymatically from either amino acid. Inhibition of the glucosyltransferase by the amino acids administered could be excluded as a possible explanation of the experimental results because the purified enzyme was not inhibited by concentrations of valine or isoleucine up to 10^{-2} M. Hence it can be concluded that both cyanoglucosides are formed *in vivo* from the corresponding cyanohydrins by the same glucosyltransferase (Fig. 1), provided that UDP-glucose is not a limiting factor *in vivo*.

TABLE 3. FEEDING EXPERIMENTS WITH SHOOTS OF 5-day-old FLAX SEEDLINGS

Expt.	Amino acid used in pretreatment	^{14}C-Cyanohydrin administered	^{14}C-Labeled glucoside isolated	(dis/min)	Control (%)
1	None	ACH	LIN	4440	100
	VAL	ACH	LIN	2900	65
	ILE	ACH	LIN	2530	57
2	None	BCH	LOT	1110	100
	VAL	BCH	LOT	400	36
	ILE	BCH	LOT	340	31

Radioactive acetone cyanohydrin (ACH) and butanone cyanohydrin (BCH) were administered after pretreatment of the plants with unlabeled valine or isoleucine. The total amount of radioactivity isolated with the glucosides linamarin (LIN) and lotaustralin (LOT) is compared with the values obtained from control experiments in which the seedlings received no pretreatment.

Similar competition experiments were carried out with two other intermediates of the linamarin pathway,[5,7] isobutyraldoxime and isobutyronitrile. Again the incorporation of label from acetone cyanohydrin and butanone cyanohydrin respectively into the cyanoglucosides was reduced when the plants were preincubated with either isobutyraldoxime or isobutyronitrile. However, these results are not conclusive, since the aldoxime also reacts chemically with the cyanide in equilibrium with a cyanohydrin, and the nitrile acts as an inhibitor of the glucosyltransferase.[8] It is interesting in this connection that the glucosylation of both cyanohydrins is increasingly inhibited by increasing concentrations of isobutyronitrile at comparable rates. Further work at the enzymatic level has to be done to establish whether the conversion of the aldoximes and nitriles corresponding to the glucosides linamarin and lotaustralin, and probably the conversion of the other intermediates as well, is also catalyzed by enzymes common for both pathways.

EXPERIMENTAL

Materials

Seed of *Linum usitatissimum* L. (linen flax) was obtained from the California Crop Improvement Office, University of California, Davis.

Uniformly ^{14}C-labeled L-amino acids were purchased from the New England Nuclear Corporation, Boston, Mass. and diluted with non-labeled material to give a specific activity of 100 μc/m-mole. ^{14}C-acetone cyanohydrin (22 μc/m-mole) and ^{14}C-butanone cyanohydrin (4 μc/m-mole), both labeled in the cyanide moiety, were prepared according to the method described previously.[5] All of the other chemicals were of reagent grade purity and purchased from various companies.

Enzymatic Glucosylation

UDP-glucose–ketone cyanohydrin β-glucosyltransferase from flax acetone powder was isolated and purified as described elsewhere.[6] Aliquots of 100 μl from each of the protein fractions listed in Table 1 were used for the enzymatic glucosylation of ^{14}C-labeled acetone cyanohydrin and butanone cyanohydrin. The incubation mixture contained 100 μmoles Tris-HCl (pH 7·6), 0·5 μmoles UDP-glucose, 2 μmoles cyanohydrin, and 100–700 μg protein in a total volume of 0·2 ml. For further details of the enzyme assay, see Ref. 6.

Feeding Experiments

Flax seedlings were grown for 4 days in the dark and then for 20 hr under continuous light. Solutions of 50 μmoles of valine or isoleucine in 0·2 ml of distilled water were administered to 10 shoots of flax seedlings for 1 hr. Then 10 μmoles of the radioactive compounds as indicated in Tables 2 and 3 were fed. After metabolism for a total period of 6 hr under artificial light, the shoots were frozen in liquid N_2, ground in a mortar, and extracted with 20 ml of boiling 80% ethanol for 10 min. The solution was filtered, the solvent evaporated, and the residue dissolved in 0·5 ml of 10% isopropanol. Aliquots of 100 μl were taken for paper chromatography in MeCOEt–Me_2CO–H_2O (15:5:3, v/v). Radioactive spots on the chromatograms were detected with a "Vanguard Automatic Chromatogram Scanner". They were cut out, and the radioactivity was measured in Bray's solution[9] with 62–68 per cent counting efficiency in a Packard "Tri-Carb" Liquid Scintillation Spectrometer.

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