The Biosynthesis of Cyanogenic Glycosides in Higher Plants

I. PURIFICATION AND PROPERTIES OF A URIDINE DIPHOSPHATE-GLUCOSE-KETONE CYANOHYDRIN β-GLUCOSYLTRANSFERASE FROM LINUM USITATISSIMUM L*

(Received for publication, September 29, 1969)

KLAUS HAHLBROCK† AND ERIC E. CONN

From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

SUMMARY

An enzyme which catalyzes the transfer of glucose from UDP-glucose to α-hydroxyisobutyronitrile (acetone cyanohydrin) has been isolated from flax seedlings (Linum usitatissimum L). The product of this reaction was shown to be identical with linamarin, one of the two cyanogenic glucosides produced by the flax plant.

The partially purified UDP-glucose-ketone cyanohydrin β-glucosyltransferase exhibited a high degree of specificity for the two aliphatic side chains of acetone and butanone cyanohydrins as well as for UDP-glucose as the glucose donor. This suggests that its natural function is to catalyze the last step in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin from the precursor cyanohydrins.

In a series of steps resulting in a purification of 120-fold, the glucosyltransferase was separated from the β-glucosidase "linamarase" which is present in the flax plant. No requirements for metal ions or sulfhydryl reagents could be shown for the glucosyl transferase.

Based on the results of feeding experiments with labeled precursors, we recently proposed a biosynthetic pathway for the formation of cyanogenic glucosides in higher plants (1). In this pathway α-hydroxynitriles (cyanohydrins) were considered to be the immediate precursors of the glucosides. The low dilution factors observed on incorporation of certain radioactive cyanohydrins into naturally occurring cyanogenic glucosides by plants which are synthesizing the glucosides indicated that the cyanohydrins were precursors. Thus in flax seedlings, acetone cyanohydrin was efficiently converted to linamarin (I) and butanone cyanohydrin was converted to lotaustralin (II)† (see Formulas I and II). In addition, the cyanogenic glucoside prunasin, which normally is not produced by the flax plant, was formed when radioactive benzaldehyde cyanohydrin was administered to the shoots of flax seedlings (2). However, the dilution factor of unity found in this case was interpreted as evidence that the product was exclusively formed from a nonendogenous precursor.

Since the flax plant possessed the ability of glucosylating cyanohydrins other than those leading to the naturally occurring glucosides, this reaction could be regarded as a detoxification process carried out by enzymes exhibiting little or no specificity. In order to find out whether there is an enzyme in flax specific for the glucosylation of the precursor cyanohydrins of linamarin and lotaustralin, we isolated and partially purified a protein fraction from flax seedlings which catalyzes the glucose transfer from UDP-glucose to acetone cyanohydrin and determined its substrate specificity. We also attempted to separate this glucosyltransferase from the hydrolytic enzyme linamarase which would interfere with the assay for the transferase reaction by hydrolyzing the enzyme product (Fig. 1).

EXPERIMENTAL PROCEDURE

Enzyme Purification

Crude Extract—Seeds of Linum usitatissimum L (linen flax) were soaked overnight in tap water and germinated in the dark for 4 days. The seedlings were then exposed to light for 24 hours and an acetone powder of the shoots was prepared. The following purification of the enzyme was then carried out at 0°C. Ten grams of acetone powder were mixed with 10 g of Polyclar AT and extracted for 60 min with 500 ml of 0.2 M Tris-HCl buffer, pH 7.6, that contained 0.5 ml of β-mercaptoethanol. The suspension was filtered through four layers of cheesecloth and centrifuged for 15 min at 17,000 × g.

Manganese Chloride and Dowex 1 Step—A solution of 1 M MnCl₂ was slowly added to the supernatant solution to give a final concentration of 0.05 M MnCl₂. After stirring for 15 min, the yellow precipitate which contained most of the nucleic acids and about one-half of the original amount of protein was removed.
by centrifugation and discarded. Solid (NH₄)₂SO₄ was added to the supernatant solution to give a final concentration of 56 g/100 ml (80% saturation). The precipitate was centrifuged at 17,000 × g and redissolved in 400 ml of 0.1 M Tris-HCl buffer, pH 7.6, containing 0.4 ml of β-mercaptoethanol. Ten grams of Dowex 1 (Cl form, equilibrated with 0.2 M Tris-HCl buffer, pH 7.6) were added, the mixture was stirred for 30 min, and the ion exchange resin was removed by filtration through glass wool.

The specific activity of the enzyme solution was increased by 50% by this treatment although only little protein was removed. This effect has been observed previously and is attributed to the adsorption of enzyme inhibitors which were not removed by dialysis or gel filtration.

Ammonium Sulfate Fractionation—The filtrate from the Dowex treatment was adjusted to 35% saturation by the addition of solid (NH₄)₂SO₄, stirred for 30 min, and then centrifuged for 10 min at 17,000 × g. Fractions of 35 to 50% and 50 to 80% saturation were obtained in the same manner. The intermediate fraction (35 to 50% saturated with (NH₄)₂SO₄) which contained 70% of the total glucosyltransferase activity was dissolved in 20 ml of the buffer used for the initial crude extract and dialyzed for 5 hours against 4 liters of 0.002 M Tris-HCl buffer, pH 7.6.

DEAE-cellulose Column Chromatography—A dialyzed protein solution was applied to a DEAE-cellulose column (2 × 27.5 cm) that had been equilibrated with 0.002 M Tris-HCl buffer, pH 7.6. The proteins were eluted with a linear Tris-HCl buffer gradient increasing from 0.002 to 1 M, pH 7.6. The gradient was obtained by mixing 500 ml each of the two buffers, 0.002 M and 1 M Tris-HCl, pH 7.6. Fractions of 5 ml each were collected and assayed for glucosyltransferase and linamarase activities.

Most of the glucosyltransferase activity, which was not separated from linamarase by this procedure, was eluted at a buffer concentration of 0.25 to 0.3 M. These fractions were combined, adjusted to 80% saturation with solid (NH₄)₂SO₄ stirred for 30 min, and centrifuged at 17,000 × g. The supernatant fraction was discarded, and the pellet was dissolved in 1.5 ml of the buffer used for the initial acetone powder extract.

Sephadex G-100 Column Chromatography—The concentrated protein solution from the DEAE-cellulose column was passed through a Sephadex G-100 column (2 × 30 cm) with 0.02 M Tris-HCl buffer, pH 7.6. One-milliliter fractions were collected, and the activity of the two enzymes, linamarase and the glucosyltransferase, was determined in these fractions. The combined fractions possessing the highest glucosyltransferase activity (e.g., Fractions 45 to 55 in Fig. 2) were used to determine the properties of the enzyme. Table I gives a typical example of the purification procedure.

Enzyme Assays

Assay for Glucosyltransferase Activity—A mixture containing 50 μmoles of Tris-HCl (pH 7.6), 0.5 μmole of UDP-glucose, 2 μmoles of acetone cyanohydrin-1-14C, and 30 to 500 μg of protein in a total volume of 0.2 ml was incubated at 30° for 60 min. The reaction was stopped by boiling the mixture for 2 min in a water bath and 100-μl aliquots were transferred into scintillation vials. The solvent and volatile cyanohydrin were evaporated in a desiccator over NaOH under reduced pressure and the radioactivity of the remaining nonvolatile products was measured in a scintillation counter.

One unit of activity is defined as the amount of enzyme that catalyzed the production of 1 μmole of product in 1 hour.

In order to examine the specificity of the partially purified enzyme for aromatic cyanohydrins (Table II, Experiment 2) and aliphatic alcohols (Table III), radioactive UDP-glucose rather than radioactive cyanohydrins was used in the assay described in the preceding paragraph. Aliquots (100 μl) were then chromatographed on Whatman No. 1 paper in Solvent System I, the radioactive peaks corresponding to the products formed were cut out, and the radioactivity was measured by scintillation counting. The products obtained in these experiments were identified by their chromatographic behavior and compared with reference compounds in those cases in which naturally occurring glucosides were available. The Rf values observed in Solvent System I for the glucosides formed from the following cyanohydrins were: acetone cyanohydrin, 0.21; acetone cyanoxydrin (linamarin), 0.54; butanone cyanohydrin (lotaustralin), 0.67; 3-pentanone cyanohydrin, 0.75; benzaldehyde cyanohydrin (prunasin), 0.82. The Rf values of the compounds formed enzymatically from UDP-glucose and acetone or butanone cyanohydrins (that is, the precursors of linamarin and lotaustralin) in Solvent Systems I, II, and III were found to be identical with those of the natural products isolated from the intact plant. Furthermore, the glucoside of acetone cyanohydrin synthesized by the purified enzyme showed the same behavior as authentic linamarin when hydrolyzed by the crude acetone powder extract under the assay conditions described below for linamarase.

Assay for Linamarase Activity—The assay was carried out in Warburg flasks containing 0.1 ml of 1 N NaOH in the center well. The reaction mixture which contained 1.2 mCi of linamarin-1-14C in 0.5 ml of 0.2 M sodium phosphate buffer, pH 6.2, and 0.2 ml of protein solutions was incubated at 30° for 16 hours. The cyanide formed upon hydrolysis of linamarin was trapped in the center well, and its radioactivity was measured in a scintillation counter.

Other Methods

Paper Chromatography—The following solvent systems were used for descending paper chromatography on Whatman No. 1, Whatman No. 3, or Whatman No. 3MM paper: Solvent I—butanone-acetone-water, 15:5:3; Solvent II—butanol-acetic acid-water, 12:3:5; Solvent III—butanol-pyridine-water, 8:4:3.

Synthesis of Cyanohydrins—The 1-14C-labeled cyanohydrins of acetone cyanohydrin and the aliphatic ketones were synthesized according to the method described previously for the preparation of acetone cyanohydrin-1-14C (1). The radioactive cyanohydrins were stored in ether solutions over anhydrous sodium sulfate and charcoal at concentrations of about 200 to 500 μM. The solvent was evaporated from aliquots of these solutions and the
radiactive cyanohydrins were dissolved in the appropriate buffer solutions immediately before use.

The specific activity of the cyanohydrins was determined by hydrolyzing approximately 10 μmoles of the 1-¹⁴C-labeled cyanohydrin in Warburg flasks with 2 ml of sodium phosphate buffer, pH 6.2, containing 100 μmolcs of semicarbazide·HCl. By incubating for 48 hours at 20° the H⁺CN liberated from the center well was trapped in 0.5 ml of 1 N NaOH. Aliquots of the center well solutions were then assayed for cyanide by the method of Aldridge (4) and for radioactivity in a scintillation counter. The specific activities of the cyanohydrins varied from 4 to 22 μCi per mmole. The results obtained from the studies on enzyme specificity were corrected for these variations.

Benzaldehyde cyanohydrin (mandelonitrile) was prepared by the slow addition of a concentrated aqueous solution of 245 mg of NaCN (5 mmole) to a suspension of 1.05 g (5 mmole) of the benzaldehyde-sodium bisulfite addition product in 2 ml of water. The reaction mixture was cooled in an ice bath. After 1 hour a yellowish layer of benzaldehyde cyanohydrin which separated from the aqueous solution was removed. The product was dried over a mixture of anhydrous sodium sulfate and charcoal.

Linamarin-¹⁴C and lotaustralin-¹⁴C labeled uniformly in the glucosyl moiety were isolated from flax shoots which were fed uniformly labeled valine-¹⁴C or isoleucine-¹⁴C according to the method described previously (5). The glucosides were purified by chromatography on Whatman No. 3MM paper in Solvents I, II, and III.

p-Glucosyloxybenzaldehyde was synthesized by the method of Abrol, Conn, and Stoker (6). Polyclar AT (insoluble polyvinyl pyrrolidone), obtained from General Aniline and Film Corporation, New York, was purified by the procedure described by Loomis and Battaile (7). UDP-glucose-¹⁴C was a gift from Dr. J. Preiss. All of the other chemicals were commercially available and were purchased from various companies.

Radioactivity was measured in Bray's solution (8) with a Packard Tri-Carb liquid scintillation spectrometer. Radioactive spots on chromatograms were detected with a Vanguard automatic chromatogram scanner.

Protein was determined by the biuret method in crude preparations (9) and by the method of Warburg and Christian (10) in purified solutions.

RESULTS

The 120-fold purification of an enzyme catalyzing the transfer of glucose from UDP-glucose to acetone cyanohydrin was achieved within 36 hours by a sequence of steps common in enzyme purification (Table I). Time is of the essence because the enzyme loses its activity rapidly, having a half-life of about 4 days. We were not able to stabilize it by the addition of various amounts of glyceral, ethanol, ammonium sulfate, dithiothreitol, or albumin or by storing it at pH 7.0 (instead of pH 7.6), at −18° or at room temperature. When Polyclar AT and β-mercaptoethanol were omitted in the initial extraction of the acetone powder, about 20% less activity was obtained. β-Mercaptoethanol could not be replaced by equimolar amounts of ascorbate. The purified enzyme is not stimulated by concentrations of β-mercaptoethanol or dithiothreitol up to 100 mM and it does not require Mg²⁺ ions. MnCl₂ was inhibitory at all concentrations tested (0.5 mM, 5 mM, and 50 mM).

During the first four steps of the purification, the glucosyltransferase is contaminated with the hydrolytic enzyme linamarase. Although the two enzymes were not separated on the DEAE-cellulose column, a separation was achieved on a Sephadex G-100 column. Here the contaminating glucosidase was eluted with the fractions containing most of the proteins, whereas the glucosyltransferase was eluted in the later fractions (Fig. 2). The remaining hydrolytic activity in these fractions can probably be attributed to contamination by hydrolytic enzymes other than linamarase.

The amount of linamarin formed from UDP-glucose and acetone cyanohydrin is directly proportional to protein and to time over a period of at least 60 min. The optimum concentration of acetone cyanohydrin appears to be greater than 30 mM (Fig. 3). Because the degree of dissociation of acetone cyanohydrin varies greatly (between 20 and 70%) in the range of concentrations shown in Fig. 3, no attempt was made to determine the Kₚ for this substrate. The effect of the concentration of UDP-glucose on the enzymic reaction is shown in Fig. 4; the enzyme is saturated with UDP-glucose at approximately 10 mM.

Although the enzyme has a pH optimum between pH 8 and 9
Biosynthesis of Cyanogenic Glycosides in Higher Plants.

I. Acetone cyanohydrin

**FIG. 3.** The effect of the concentration of acetone cyanohydrin on the enzymic synthesis of linamarin. Conditions were those of the assay described in the text.

![Graph showing the effect of acetone cyanohydrin concentration on linamarin synthesis.](image1)

II. UDP-glucose

**FIG. 4.** The effect of the concentration of UDP-glucose on the enzymic synthesis of linamarin. Conditions were those of the assay described in the text.

![Graph showing the effect of UDP-glucose concentration on linamarin synthesis.](image2)

III. pH and buffers

**FIG. 5.** The effect of pH and different buffers on the enzymic synthesis of linamarin. The assay conditions were described in the text; the final concentration of the buffer was 0.2 M in every case. ○, Na₂HPO₄-citric acid; △, Tris-HCl; ×, glycine-NaOH.

![Graph showing the effect of pH and different buffers on linamarin synthesis.](image3)

and is more active in glycine-NaOH than in Tris-HCl buffer (Fig. 5), all studies on the enzyme were carried out in Tris-HCl buffer, pH 7.6, for two reasons. First, cyanohydrins dissociate increasingly rapidly at higher pH and the comparison of different cyanohydrins as enzyme substrates would be complicated by different rate constants for their dissociation. The cyanohydrins of benzaldehyde and p-hydroxybenzaldehyde also undergo the benzoin condensation in basic solutions. Second, Tris-HCl buffer partially inhibits at lower pH values the enzymic hydrolys.

**Fig. 5.** The effect of pH and different buffers on the enzymic synthesis of linamarin. The assay conditions were described in the text; the final concentration of the buffer was 0.2 M in every case. ○, Na₂HPO₄-citric acid; △, Tris-HCl; ×, glycine-NaOH.

ABOO...E coo 6

3 8 7 8 9 IO

PH

![Graph showing the effect of pH and different buffers on linamarin synthesis.](image4)

A. E. Conn, unpublished results.

The purified glucosyltransferase exhibits a high specificity for UDP-glucose. Within the experimental error, no linamarin was formed with adenosine, cytidine, guanosine, inosine, or thymidine 5'-diphosphoglucose as the glucose donor.

With regard to the cyanohydrin substrate, the enzyme has a high specificity for the 2 alkyl residues of the ketone cyanohydrins. Thus, when the radioactive cyanohydrins listed in Table II, Experiment 1, were incubated with UDP-glucose, acetone, butanone, and 3-pentanone cyanohydrins were readily converted to the corresponding glucosides. Substrates with hydrogen (acetaldehyde cyanohydrin) or with alkyl residues larger than an ethyl group reacted more slowly (3-hexanone cyanohydrin) or not at all (4-heptanone cyanohydrin). In another experiment (Table II, Experiment 2) with benzaldehyde and p-hydroxybenzaldehyde cyanohydrins, these aromatic compounds were found to be extremely poor substrates.

It should be mentioned that the cyanohydrin of p-hydroxybenzaldehyde was converted to a product which also was obtained in approximately the same amounts when p-hydroxybenzaldehyde itself was used as a substrate. The RF values of these two products in Solvent I were identical with that of p-glucosyloxybenzaldehyde (RF = 0.58). This suggests that the partially purified enzyme is contaminated with a different glucosyl transferase capable of glucosylating aromatic hydroxyl groups.

In another experiment (Table III) several alcohols were compared as glucosyl acceptors to find out whether the nitrile group

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A. E. Conn, unpublished results.
The standard assay as described in the text was used. In Experiment 1, radioactive cyanohydrins and unlabeled UDP-glucose were used. In Experiment 2, radioactive UDP-glucose and unlabeled cyanohydrins were used.

<table>
<thead>
<tr>
<th>Experiment and cyanohydrin of</th>
<th>Cyanogenic glucoside formed</th>
<th>Relative conversion (acetone cyanohydrin = 100)</th>
</tr>
</thead>
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<tr>
<td>Acetaldehyde</td>
<td>83</td>
<td>24</td>
</tr>
<tr>
<td>Acetone</td>
<td>340</td>
<td>100</td>
</tr>
<tr>
<td>Butanone</td>
<td>400</td>
<td>144</td>
</tr>
<tr>
<td>3-Pentanone</td>
<td>425</td>
<td>125</td>
</tr>
<tr>
<td>3-Hexanone</td>
<td>90</td>
<td>26</td>
</tr>
<tr>
<td>4-Heptanone</td>
<td>285</td>
<td>100</td>
</tr>
<tr>
<td>2-Acetone</td>
<td>161</td>
<td>57</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table II
Enzyme specificity for cyanohydrins

The standard assay as described in the text was used. Radioactive UDP-glucose and unlabeled alcohols were used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>R1</th>
<th>R2</th>
<th>Glucoside formed</th>
<th>Relative conversion (acetone cyanohydrin = 100)</th>
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<tbody>
<tr>
<td>Acetone cyanohydrin</td>
<td>CH3</td>
<td>CN</td>
<td>285</td>
<td>100</td>
</tr>
<tr>
<td>Ethanol</td>
<td>H</td>
<td>H</td>
<td>52</td>
<td>18</td>
</tr>
<tr>
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<td>H</td>
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<td>tert-Butanol</td>
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<td>CH3</td>
<td>90</td>
<td>32</td>
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<td>H</td>
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<td>75</td>
</tr>
<tr>
<td>2-Methyl-2-butanol</td>
<td>CH3—CH3</td>
<td>CH3</td>
<td>161</td>
<td>57</td>
</tr>
</tbody>
</table>

Table III
Enzyme specificity for nitrile group of cyanohydins

The standard assay as described in the text was used. Radioactive UDP-glucose and unlabeled alcohols were used.

DISCUSSION

Although feeding experiments with intact plants have strongly supported the role of cyanohydrins as intermediates in the biosynthetic formation of cyanogenic glucosides (1), conclusive information about the glucosylation reaction involved in the proposed pathway could only be achieved by providing further evidence at the enzymatic level. It was the purpose of this work to show that there is an enzyme in the flax plant which is responsible for the specific conversion of acetone cyanohydrin to its naturally occurring glucoside, linamarin.

The results obtained from experiments with the 120-fold purified protein fraction show that the transglucosylation reaction is confined to a limited number of aliphatic ketone cyanohydrins and other alcohols with a similar chemical structure. Among the compounds tested, acetone, butanone, and 3-pentanone cyanohydrins were the substrates most rapidly converted to the corresponding glucosides. The enzyme has a distinct specificity for the alkyl residues in the two aliphatic side chains as well as for the nitrile group. It also seems to be absolutely specific for UDP-glucose as the glucose donor. The hydrolysis of the glucoside formed in vitro from UDP-glucose and acetone cyanohydrin by linamarase, a β-glucosidase, shows that the enzyme product is the β-anomer. Butler, Bailey, and Kennedy (11) and Clapp et al. (12) have established the β-configuration of linamarin. The purified glucosyltransferase therefore is a UDP-glucose:ketone cyanohydrin β-glucosyltransferase (EC 2.4.1).

A number of enzymes have been isolated from plant tissues which catalyze the transfer of glucose from UDP-glucose to aliphatic (13–17) or aromatic (18–20) hydroxyl groups. The properties of the flax enzyme do not differ basically from those described for the transglucosylases mentioned above, although most of these have not been purified as extensively. The high pH optimum for the glucosylation of cyanohydrins is in agreement with the observation of Yamaha and Cardini who found an optimum of pH 6.5 for the glucosylation of an aromatic hydroxyl group (18) and an optimum between pH 8 and 9 for the glucosylation of an aliphatic hydroxyl group (15). The relatively poor, but nevertheless significant, glucosylation of benzoic acid cyanohydrin (Table II) could account for the formation of prunasin which was observed when the nonendogenous cyanohydrin was fed to flax seedlings (2), although other nonspecific glucosylating enzymes might also have formed the glucoside.

Although the addition of β-mercaptoethanol to the buffer solution during the early steps of the enzyme purification increased the yield of enzyme activity, sphydroxyl reagents at concentrations between 0.01 and 100 mM had no effect on the purified enzyme. This is in contrast to the findings of Kleinhofs, Haskins, and Gorz (20) who reported that the glucosylation of ortho-hydroxy-trans-cinnamic acid in cell-free extracts of Melilotus albus had an absolute requirement for β-mercaptoethanol or cysteine. Because of the rapid dissociation of the cyanohydrins toward an equilibrium with the ketone (or aldehyde) and cyanide, the K_m values of these substrates were not determined. For UDP-glucose, a K_m of about 1 mM was found.

An important and so far unanswered question is whether or
not the formation of linamarin and lotaustralin in vivo is catalyzed
by the same enzyme. During the purification of this glucosyl-
transferase no indication was found for the presence of another
enzyme which would also glucosylate acetone cyanohydrin. If
the isolated UDP-glucose-ketone cyanohydrin β-glucosyltrans-
ferase were only one of two different enzymes, one would expect
to find a second protein fraction during one of the purification
steps with probably a similar substrate specificity. Since this
was not the case, it is possible that either the purified protein
fraction contains a mixture of an acetone cyanohydrin and a
butanone cyanohydrin-glucosylating enzyme, or that both of
these cyanohydrins are glucosylated by the same enzyme.
Further studies are being made to answer this question.

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