Prunasin Biosynthesis by Cell-Free Extracts from Black Cherry (*Prunus serotina* Ehrh.) Fruits and Leaves

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Immature fruits and leaves of black cherry (*Prunus serotina* Ehrh.) accumulate the cyanogenic glucoside prunasin (the β-glucoside of (*R*)-mandelonitrile). Cell-free extracts from these tissues catalysed the stereospecific glucosylation of (*R,S*)-mandelonitrile to (*R*)-prunasin at rates of 0.2–2.0 μmol/h/mg protein. Uridine diphosphate glucose ($K_m = 0.32$ mM) acted as glucose donor. The optimum concentration of (*R,S*)-mandelonitrile was 20 mM. Highest activity was exhibited at pH 7.0–8.0 in Tris-phosphate buffer, and no additional cofactors were required. β-Mercaptoethanol (14.5 mM), provided in the homogenization medium to prevent browning of homogenates, did not stimulate the rate of prunasin production. In addition to (*R*)-mandelonitrile (100%), significant activity was shown towards mandelamide (21%), benzyl alcohol (15%), mandelic acid (8%) and benzoic acid (153%), but not towards prunasin. Mandelonitrile glucosyltransferase activity was most stable at – 20 °C in the presence of 10% glycerol.

Introduction

The most important cause of cyanide poisoning among domestic animals is ingestion of such plants as arrow grass (*Triglochin* sp.), sorghum (*Sorghum bicolor*), wild black cherry (*Prunus serotina*), choke cherry (*P. virginiana*), and flax (*Linum usitatissimum*) [1]. These plants contain cyanogenic glycosides that, when hydrolysed by enzymes during mastication and digestion, yield hydrocyanic acid (HCN). This phenomenon of cyanogenesis is especially common among members of the Rosaceae, where several species possess one or more of three cyanogenic glycosides derived from L-phenylalanine, namely prunasin, sambunigrin, and the disaccharide amygdalin. Whereas the catabolism of prunasin (the β-glucoside of (*R*)-mandelonitrile) and amygdalin (the β-gentiobioside of (*R*)-mandelonitrile) has been extensively studied [2–4], our knowledge concerning the biosynthesis of these cyanogens is currently restricted to *in vivo* isotopic evidence. Nevertheless, the successful incorporation of L-phenylalanine, phenylacetaldoxime, phenylacetonitrile, and mandelonitrile into prunasin by cherry laurel (*P. laurocerasus*) and peach (*P. persica*) shoots strongly supports the proposed biosynthetic pathway shown in Fig. 1, in which mandelonitrile plays a central role [5–7].

As part of our goal to verify this pathway at the enzymic level, we report here the presence in cell-free extracts from immature fruits and young leaves of black cherry (*P. serotina*) of a soluble glucosyltransferase which glucosylates mandelonitrile to prunasin.

Abbreviations: UDPG, uridine diphosphate glucose; PVP, polyvinylpolypyrrolidone; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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Materials and Methods

Chemicals and chromatographic materials

Uridine diphosphate-D-[U-^{14}C]glucose (330 mCi/mmol) was purchased from New England Nuclear, Boston, MA., and diluted with unlabelled UDPG as required. (*R,S*)-Mandelonitrile, prunasin, polyvinylpolypyrrolidone, almond emulsin, Sigma-Sil-A, and UDPG were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade or better. Cellulose and silica gel IB-F thin layer chromatography sheets were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ.

Plant materials and analysis of their cyanogen content

Young leaves and immature fruits of black cherry (*Prunus serotina* Ehrh.) were gathered from Hickory Hill Park, Iowa City, during the first 10 weeks following fertilization of the flowers (May to mid-

July 1982). During these studies, the cherries became increasingly difficult to homogenize using a pestle and mortar due to the woodiness of the developing endocarp. To analyze qualitatively the cyanogen content, plant material was ground in liquid nitrogen in a mortar and, while still frozen, added to boiling methanol. After 10 min extraction, the plant debris was removed by filtration. The filtrate was concentrated by rotary evaporation and chromatographed alongside authentic samples of prunasin and amygdalin on Whatman 3MM paper using solvent system I. After thoroughly drying the paper, the cyanogens were located by the Feigl-Anger "sandwich" technique [8], employing almond emulsin to release HCN.

Enzyme purification

All stages were carried out at 4 °C. Black cherry tissue (weighing 5.5 g) was homogenized with 10–15 ml of buffer I, 0.55 g PVP, and some quartz sand in a mortar. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at $12000 \times g$ for 25 min. An aliquot (2.5 ml) of the supernatant liquid was chromatographed on a Sephadex G-25 column (1.5 × 8.3 cm), which had been pre-equilibrated with buffer II. Elution was carried out with buffer II, and the eluate was collected for assay of glucosyltransferase activity and protein content.

Buffer solutions

The following buffer solutions were used: (I) 0.2 M Tris-HCl buffer, pH 8.0, containing 14.5 mM β-mercaptoethanol; (II) 20 mM Tris-HCl buffer, pH 8.0.

Glucosyltransferase assay

The standard assay mixture for O-glucosyltransferase activity contained 3 µmol (*R,S*)-mandelonitrile (in 10 µl methanol), 300 nmol uridine diphosphate-D-[U-^{14}C]glucose (containing 52 nCi), 30 µmol Tris-phosphate buffer, pH 7.33, and up to 30 µg protein in a total volume of 0.15 ml. Control reaction vessels, in which mandelonitrile was omitted, were included where appropriate. After incubation at 30 °C for 20–60 min, the reaction was terminated by heating the reaction mixture at 95 °C for 2 min. Descending paper chromatography on Whatman 3MM paper with solvent system I was used to analyze reaction products. The product zones were cut out and counted in a Beckman LS-100C scintillation counter using 5 ml of Andersons scintillation fluid (0.3% PPO and 0.02% POPOP in xylene-Triton X-114 (3:1, by vol.)).

Chromatographic identification of reaction products

Identification of the reaction product of mandelonitrile glucosylation was made by co-chromatography with an authentic (*R*)-prunasin sample on Whatman 3MM paper using solvent system I, on cellulose TLC sheets with systems II–V, and on silica gel TLC sheets with systems VI–VII. The following solvent systems were utilized: (I) *n*-butanol-acetic acid-H_2O, 4-1-5, upper phase; (II) *n*-propanol-H_2O, 70-30; (III) methanol-H_2O, 90-10; (IV) ethyl acetate-acetone-H_2O, 32-40-8; (V) *n*-butanol-ethanol-H_2O, 7-2-2; (VI) chloroform-methanol, 5-1; (VII) water-saturated *n*-butanol.

Since these systems fail to resolve (*R*)-prunasin and (*S*)-sambunigrin, the reaction product was further identified using gas liquid chromatography. Samples of the reaction products, (*R*)-prunasin and an (*R*)-prunasin/(*S*)-sambunigrin racemic mixture were dried and treated with Sigma-Sil-A (hexamethyldisilazane and trimethylchlorosilane in pyridine). The trimethylsilyl derivatives were resolved in a nickel column (72 in. × 0.25 in. inside diameter) packed with 3% (w/w) Dexsil 300 on Gas Chrom Q (100 to 200 mesh) and detected by thermal conductivity. The oven (Hewlett Packard 5710) was heated from 200 °C to 250 °C at 2° or 4 °C per min with a carrier gas flow (helium) of 65 ml per min. Analysis of radioactive products was undertaken using a Packard 894 proportional counter (30–40% efficiency).

Protein estimation

The protein content of leaf and cherry extracts was determined by the Bradford procedure [9], using crystalline bovine serum albumin as standard.

Results and Discussion

Undoubtedly the best known of all cyanogenic glycosides is amygdalin, which, in addition to being the first cyanogen to be isolated and fully characterized [10], was in recent years promoted without

foundation as an anticancer agent [11]. Amygdalin accumulates in the seeds of several rosaceous species, while its monosaccharide derivative prunasin is found in the vegetative portions of these plants [12, 13]. These cyanogenic glycosides are apparently responsible for numerous cases of acute cyanide poisoning of man and his livestock following the ingestion of seeds of bitter almonds, apricots, choke cherries, and leaves of peaches [11]. In view of their toxicity, these compounds have received much attention, especially with respect to their catabolism to hydrogen cyanide [2, 14]. By contrast, much less is known about the biosyntheses of these toxins or about the genes controlling their formation [15]. Radioisotopic labelling studies [5–7] have provided strong evidence for the biosynthetic pathway shown in Fig. 1, but it has not yet been confirmed at the enzymic level. In analogy with the biosyntheses of dhurrin, linamarin and taxiphyllin [16], it is expected that a microsomal system would convert L-phenylalanine to (*R*)-mandelonitrile. In the leaves and fruits, prunasin would be produced by the glucosylation of (*R*)-mandelonitrile. The further glucosylation of prunasin to amygdalin would occur in ripening fruits, presumably catalysed by a distinct glucosyltransferase.

In efforts to provide evidence at the enzymic level for the proposed biosynthetic pathway, we have searched for UDPG:mandelonitrile glucosyltransferase activity in immature fruits and young leaves of black cherry (*P. serotina*).

Cyanogen content of leaves and immature fruits

In general, this study utilized young leaves from the first three nodes only. Qualitative analysis showed that these leaves, which were the most active in releasing HCN as judged by the Feigl-Anger test, contained the cyanogen prunasin as previously reported [12, 13]. The immature fruits differed from the leaves in two aspects of cyanogenesis. Firstly, the unripened fruits (which were still green in colour at this stage of maturation) contained both prunasin and amygdalin in the approximate ratio 85:15 when extracted as described in the Methods section. Identical results were obtained if the cherries were placed in boiling water for 5 min to inactivate β-glucosidases prior to extraction with methanol. The occurrence of both prunasin and amygdalin and their possible interconversion during the ripening period have been reported for immature fruits of *Prunus avium* [17] and *Cotoneaster* species [18]. Secondly, when the pits and pericarp were macerated either separately or together in the Feigl-Anger test, they ellicited only a weak response. A strong response, especially with the pits, could be obtained if almond emulsin (in acetate buffer) was then added to the macerates; this strongly suggests that the fruits at this stage possess relatively low amygdalin hydrolase and prunasin hydrolase activities and thus represent a suitable tissue to search for UDPG:mandelonitrile glucosyltransferase activity.

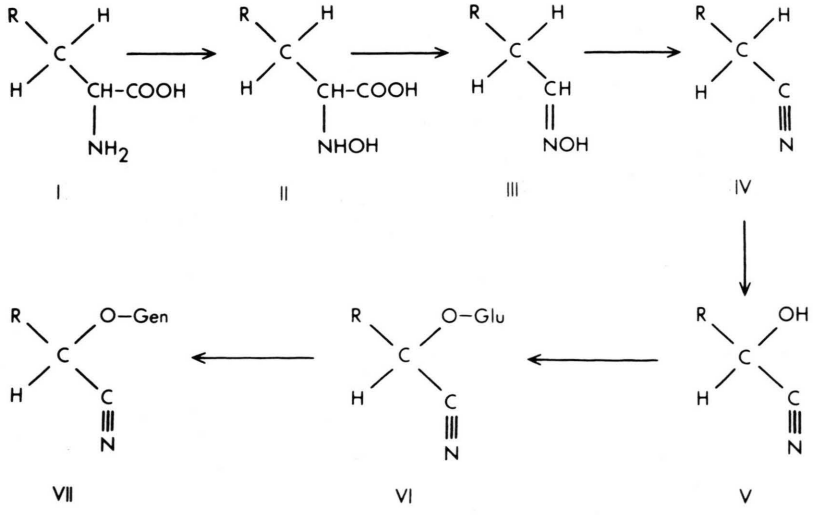

Fig. 1. The proposed biosynthetic pathway from L-phenylalanine (I) to (*R*)-prunasin (VI) and (*R*)-amygdalin (VII) in *Prunus* species. The assumed intermediates are N-hydroxyphenylalanine (II), phenylacetaldoxime (III), phenylacetonitrile (IV), and mandelonitrile (V), (R = Phenyl).

Extraction and characterization of UDPG:mandelonitrile glucosyltransferase from immature fruits

A crude preparation possessing UDP-glucose: mandelonitrile glucosyltransferase activity was obtained by maceration of immature black cherry fruits with Tris-HCl buffer and quartz sand in a mortar, followed by filtration and centrifugation. β-Mercaptoethanol (14.5 mM) was routinely added to the homogenization medium, since browning of homogenates and lower glucosyltransferase activity were recorded in its absence. Removal of phenolics was effected during homogenization by PVP and by subsequent gel filtration. The resultant filtrate catalyzed the rapid glucosylation of (*R,S*)-mandelonitrile to prunasin at rates of 0.5–2 µmol/h/mg protein. Prunasin production was not observed when mandelonitrile was omitted from the reaction mixture. In contrast to the behaviour of the *o*-coumaric acid glucosyltransferase from *Melilotus alba* [17], β-mercaptoethanol did not stimulate the rate of prunasin production and was removed at the gel filtration stage.

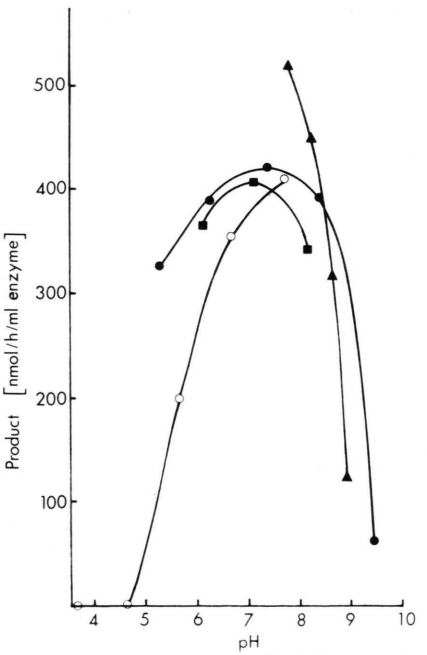

Fig. 2. Effect of pH on UDPG:mandelonitrile glucosyltransferase activity. Mandelonitrile and UDPG were incubated with the enzyme preparation as described under Materials and Methods, using 200 mM concentrations of the following buffers: K_2HPO_4-citrate (○—○), Tris-KH_2PO_4 (●—●), K_2HPO_4–KH_2PO_4 (■—■) and Tris-HCl (▲—▲).

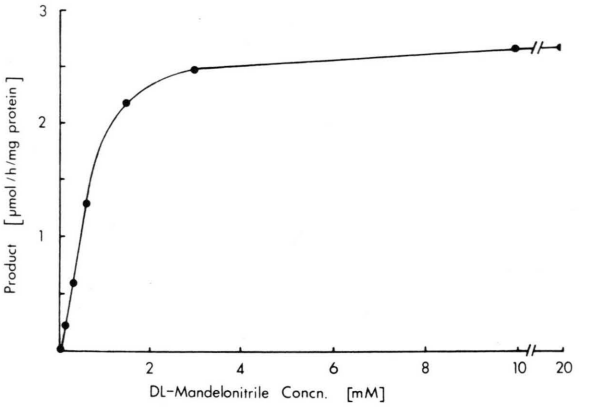

Fig. 3. The effect of DL-mandelonitrile concentration upon the rate of prunasin synthesis. Conditions were as described in the text.

The optimum pH for the glucosylation of mandelonitrile was determined using several different buffers (Fig. 2). Prunasin synthesis proceeded most rapidly between pH 7.0 and 8.0, but over 50% of this maximum rate was realized over the range between 5.8 and 9.3. By comparison, pH optima of 8.2–8.5 and 8–9 were reported for the UDPG: aldehyde cyanohydrin glucosyltransferase from sorghum [20] and the UDPG:ketone cyanohydrin glucosyltransferase from linen flax [21], respectively. Although highest rates were realized here in Tris-HCl buffer at pH 7.5, reactions were routinely undertaken in Tris-phosphate buffer, pH 7.33, in view of its superior buffering capacity. The rate of mandelonitrile glucosylation at pH 7.33, catalysed by 28 µg of enzyme extract, was linear at this pH for at least 60 min at 30 °C. The extent of prunasin production at this pH was proportional to the protein amount added up to at least 28 µg of the enzyme preparation.

Several attempts to determine the Michaelis constant for (*R,S*)-mandelonitrile were unsuccessful due to the rapid lability of this compound in alkaline media. Similar difficulties were reported previously for the cyanohydrin substrates for the sorghum and flax enzymes. However, as shown in Fig. 3, the optimum concentration of (*R,S*)-mandelonitrile was 20 mM; substrate inhibition was observed at 50 mM concentration, when only 74% of the maximum rate was realized. The apparent K_m for UDPG was 0.32 mM, when assayed at pH 7.33 in the presence of an optimum concentration (20 mM) of (*R,S*)-mandelonitrile (Fig. 4). This value is

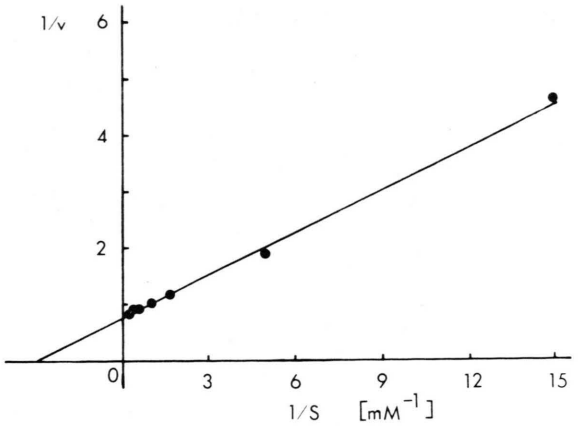

Fig. 4. Lineweaver-Burk plot of the effect of the concentration of UDP-glucose on the enzymic synthesis of prunasin.

approximately ten-fold the K_m shown by the sorghum glucosyltransferase for UDPG with *p*-hydroxymandelonitrile as glucose acceptor [20].

The product of the enzymic glucosylation of mandelonitrile co-chromatographed with an authentic sample of prunasin using 7 solvent systems as described in the Methods section. However, it should be noted that these systems cannot resolve prunasin and its epimer sambunigrin. Since mandelonitrile exists in aqueous solution as a mixture of enantiomeric cyanohydrins, it was critical to establish whether one or both of the enantiomers were glucosylated under test conditions. Using gas chromatographic analysis, it was shown that, when offered (*R,S*)-mandelonitrile as substrate, the black cherry O-glucosyltransferase exclusively utilized the (*R*)-enantiomer forming prunasin. This strict stereospecificity of the enzyme *in vitro* correlates well with the presence of prunasin and absence of sambunigrin in black cherry [13]. The cyanohydrin O-glucosyltransferases from sorghum [20] and arrow grass [22], but not from flax [21], display similar stereospecificities towards one enantiomer.

The substrate specificity of the enzyme preparation was investigated by incubating various potential acceptors, supplied at 10 mM concentration, with the enzyme at pH 7.33. Table I indicates that (*R,S*)-mandelonitrile was a far superior substrate than the closely-related compounds mandelamide and mandelic acid; that the glucosyltransferase might require the nitrile group to display high activities is further suggested by its low glucosylation rate with benzyl alcohol. Interestingly, the enzyme preparation glucosylated benzoic acid at high rates. The product β-D-glucopyranose-1-benzoate, which had not hitherto been reported in Angiosperms, has recently been isolated in high yield (488 mg/600 g fresh weight) from black cherry leaves [23]. Benzoic acid, released from glucose-1-benzoate and prunasin (after oxidation of benzaldehyde) during the natural senescence of black cherry leaves, inhibited the growth of red maple seedlings in concentrations as low as 0.1 mM [24].

The accumulation of amygdalin in ripe fruits of the domesticated rosaceous species has long been known [10, 11]. This fact poses the question whether ripening fruits are able to synthesize amygdalin from L-phenylalanine or whether prunasin is synthesized in the vegetative organs and is translocated to the ripening fruits, where it becomes glucosylated yielding amygdalin. Although by itself not conclusive, our demonstration of UDPG:mandelonitrile glucosyltransferase in immature fruits supports the former possibility. Importantly, extracts prepared from fruits at this stage of development were unable to glucosylate prunasin to amygdalin under test conditions; this suggests that amygdalin production is controlled by a distinct glucosyltransferase which appears later in fruit maturation.

Stability experiments indicated that the mandelonitrile glucosyltransferase was most stable at − 20 °C in the presence of 10% glycerol. In the absence of glycerol, only 11% and 6% of the initial glucosyltransferase activity remained after 2 days at 4 °C and − 20 °C, respectively; with glycerol, 28% and 72%, respectively, remained at these temperatures.

Table I. Substrate specificity of *Prunus serotina* glucosyltransferase preparation. O-Glucosyltransferase activity was assayed at pH 7.33 as described in the Methods section, but using 10 mM concentration of glucose acceptors.

Substrate	Specific activity [μmol/h/mg/protein]	% Activity (mandelonitrile = 100%)
(*R,S*)-Mandelonitrile	0.70	100
Mandelamide	0.15	21
Mandelic Acid	0.06	8
Benzyl alcohol	0.10	15
Benzoic acid	1.07	153
(*R*)-Prunasin	n.d.	n.d.

n.d.: not detectable.

UDP-Glucose:mandelonitrile glucosyltransferase activity in cherry leaf preparations

Leaf extracts catalysed the glucosylation of (*R,S*)-mandelonitrile at rates of 0.2–1.25 μmol/h/mg protein; the relative lower rates may reflect the greater β-glucosidase activities in this tissue. The product of glucosylation co-chromatographed with an authentic sample of prunasin using 7 paper and thin-layer chromatography systems as described earlier. In addition to (*R,S*)-mandelonitrile, significant glucosyltransferase activity was shown towards benzyl alcohol and mandelamide, but not towards prunasin. Stability experiments indicated that the enzyme retained most activity when stored at −20 °C in the presence of 10% glycerol. Whereas the leaf extracts had lost all glucosyltransferase activity after storage for 4 days at 4 °C and −20 °C, 11% and 87% of the initial activities were retained when the enzyme preparations were stored with 10% glycerol at these same temperatures.

In conclusion, this paper, by describing kinetic properties of the UDPG:mandelonitrile glucosyltransferase activity in black cherry fruits and leaves, has provided confirmation for the central role of mandelonitrile in the proposed biosynthetic pathway to prunasin and amygdalin. Purification and characterization of this glucosyltransferase are in progress in this laboratory. Furthermore, ongoing searches are being made in extracts from developing fruits for a glucosyltransferase which converts prunasin into amygdalin.

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