Development of the Potential for Cyanogenesis in Maturing Black Cherry (Prunus serotina Ehrh.) Fruits

Elisabeth Swain, Chun Ping Li, and Jonathan E. Poulton*
Department of Botany, University of Iowa, Iowa City, Iowa 52242

ABSTRACT

Biochemical changes related to cyanogenesis (hydrogen cyanide production) were monitored during maturation of black cherry (Prunus serotina Ehrh.) fruits. At weekly intervals from flowering until maturity, fruits (or selected parts thereof) were analyzed for (a) fresh and dry weights, (b) prunasin and amygdalin levels, and (c) levels of the catabolic enzymes amygdalin hydrolase, prunasin hydrolase, and mandelonitrile lyase. During phase I (0–28 days after flowering [DAF]), immature fruits accumulated prunasin (mean: 3 micromoles/fruit) but were acyanogenic because they lacked the above enzymes. Concomitant with cotyledon development during mid-phase II, the seeds began accumulating both amygdalin (mean: 3 micromoles/seed) and the catabolic enzymes and were highly cyanogenic upon tissue disruption. Meanwhile, prunasin levels rapidly declined and were negligible by maturity. During phases II (29–65 DAF) and III (66–81 DAF), the pericarp also accumulated amygdalin, whereas its prunasin content declined toward maturity. Lacking the catabolic enzymes, the pericarp remained acyanogenic throughout all developmental stages.

Over 2650 plant species distributed throughout 130 families of angiosperms, gymnosperms, and ferns release large quantities of HCN upon tissue disruption or infection (22). Most frequently, cyanogenesis (HCN production) arises during the degradation of cyanogenic glycosides (19). Because tissue maceration is required for large-scale cyanoglycoside catabolism, it is generally assumed that these glycosides and their respective catabolic enzymes are compartmentalized in intact plants at either tissue or subcellular levels (18). Such compartmentation of the components constituting the “cyanide bomb” prevents any premature HCN release until tissue disruption allows their interaction. Given the well-documented toxicity of HCN (17), a role for cyanogenesis in plant protection against herbivores, pathogens, and competitors is appealing and has received some experimental support (8, 16). However, one should keep in mind the possibility that the carbonyl compounds released during cyanoglycoside catabolism may also act as feeding deterrents (8). Recent studies with germinating seedlings have shown that cyanogenic glycosides and the related cyanolipids may additionally serve as nitrogen storage compounds (24, 25).

The biochemistry of cyanogenic glycoside accumulation by maturing seeds and fruits has so far received little attention (4, 5, 7, 13–15). Cyanoglycoside levels have been monitored during fruit development but, without exception, they were not correlated with changes in levels of enzymes involved in glycoside metabolism. The highly cyanogenic nature of rosaceous stone fruits (e.g. almonds, peaches, cherries) has long been known. Although the fleshy portions of these fruits are traditionally regarded as acyanogenic and therefore innocuous, the seeds, which accumulate the cyanogenic disaccharide (R)-amygdalin, have been responsible for numerous cases of acute cyanide poisoning of humans and domesticated and wild animals (17). For several years, our laboratory has been investigating amygdalin metabolism in black cherry seeds (10, 11, 30). Upon crushing mature seeds, amygdalin is rapidly catabolized to HCN, benzaldehyde, and glucose through the sequential action of the enzymes AH, PH, and MDL. To provide further insight into the possible physiological roles played by cyanogenic glycosides, we have followed the development of the potential for cyanogenesis in maturing black cherry fruits. Levels of the cyanogenic glycosides amygdalin and prunasin and of the enzymes AH, PH, and MDL were monitored from shortly after flowering until maturity. Wherever possible, analysis was performed on separated fruit tissues to provide greater insight into developmental processes.

MATERIALS AND METHODS

Chemicals and Chromatographic Materials

(R)-Amygdalin, (R)-prunasin, polyvinylpolypyrrolidone, protease inhibitors, almond emulsin (type G-0395), horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, and glucose oxidase-peroxidase reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Chromatographic materials were purchased from the following sources: Sephadex G-25, Pharmacia Fine Chemicals (Piscataway, NJ); DEAE-cellulose, Whatman Ltd. (Kent, UK).

Plant Materials

A single black cherry (Prunus serotina Ehrh.) tree, located at Hickory Hill Park, Iowa City, was selected as source of plant materials. Full flowering occurred on May 21, 1990. Beginning 14 DAF and continuing at 7-d intervals until full maturity, developing fruits were collected between 9:00 and 10:00 AM. Samples were enclosed in plastic bags and kept on ice during transfer to the laboratory, where they were analyzed...
without delay for fresh and dry weights and for glycoside and enzyme levels.

**Analysis of Fresh and Dry Weights**

On each sampling date, the fresh weight was determined by counting and weighing a representative sample of fruits immediately upon their arrival in the laboratory. For dry weight analysis, a known number of cherries was weighed before being dried at 80°C until constant weight was attained. After endocarp development began (first noted 28 DAF), cherries were sliced in half with a razor blade to facilitate drying.

**Analysis of AH, PH, and MDL Levels by Enzymatic Assay**

At early developmental stages (0–21 DAF), enzyme levels were determined in extracts derived from whole fruits as described below. After endocarp development began (28 DAF), fruits were dissected before homogenization into two fractions, namely the developing seeds (i.e., the contents of the endocarp) and the remaining tissues (the pericarp), which were processed separately. Plant tissue (4 g, derived from a known number of cherries) was homogenized at 4°C in a mortar with 0.2 g polyvinylpolypyrrolidone, 1 g sand, and 20 mL of 0.1 M histidine-HCl buffer, pH 6.0, containing 1 mM PMSF, 5 mM EDTA, and 1 mM tosyl-lysine chloromethylketone. The homogenate was centrifuged at 17,600 g for 25 min, and the pellet was discarded. An aliquot (7–20 mL) of the supernatant liquid was chromatographed on a Sephadex G-25 column (40 × 2.5 cm) that was prequillibrated and eluted with 20 mM sodium acetate buffer, pH 5.0. Fractions containing protein (monitored at 280 nm by an Isco V4 absorbance detector) were pooled and used for estimation of PH, MDL, and protein levels. For analysis of AH levels, AH and PH were resolved by applying an aliquot (4 mL) of the G-25 eluate to a DEAE-cellulose column (2.5 × 1 cm) that had been prequillibrated with 20 mM sodium acetate buffer, pH 5.0. AH was not retained by that matrix and was eluted by washing the column with 20 mL of this buffer (11). To confirm that ion-exchange chromatography had successfully removed PH, the eluate was routinely assayed for PH activity.

β-Glucosidase activity toward amygdalin (AH activity) and prunasin (PH activity) was assayed in triplicate as described by Kuroki and Poulton (10, 11). MDL activity was assayed as previously described (30). Values shown represent the mean of all trials.

**Analysis of AH, PH, and MDL Levels by Immunoblotting**

Crude seed homogenates containing AH, PH, and MDL were prepared as described above and, after 1:1 dilution with 57.5 mM Tris-acetate, pH 4.8, containing 40% (v/v) glycerol, were stored at −20°C until processed. Equal volumes were subjected to SDS-PAGE on 10% gels (21). Proteins were electroblotted at 23°C for 2 h onto nitrocellulose filters in 25 mM Tris, 200 mM glycine, 20% (v/v) methanol transfer buffer using a Trans-Blot apparatus (Bio-Rad) at 60 V. After rinsing the blot three times (10 min each) with TBS (10 mM Tris-HCl, pH 7.4, containing 0.9% [w/v] NaCl), nonspecific protein binding sites were blocked by incubating filters for 30 min at 23°C with blocking buffer (5% [w/v] nonfat dry milk powder and 0.05% [v/v] Tween 20 in TBS). The blot was then transferred to fresh blocking buffer for another 90 min before overnight incubation with primary antibody (for origin and dilutions, see below). Unbound primary antibodies were removed by three washes (10 min each) with blocking buffer. The filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (diluted 1:3000 with blocking buffer) for 1 to 4 h at 23°C followed by three washes (10 min each) with TBS. The locations of antigen-antibody complexes were visualized by incubating with 0.05% (w/v) 4-chloro-1-naphthol and 0.05% (v/v) H2O2 in TBS containing 16.7% (v/v) methanol.

Polyclonal antibodies were raised in New Zealand White rabbits against trifluoromethanesulfonic acid deglycosylated AH, PH, and MDL (27) and purified by affinity chromatography where necessary. Their monospecificity was confirmed by western immunoblotting analysis after resolving total seed proteins by one-dimensional and two-dimensional SDS-PAGE (for anti-MDL antibodies, see ref. 29; for anti-AH and anti-PH antibodies, data to be published elsewhere). Before use, anti-AH, -PH, and -MDL antibodies were diluted 1:500, 1:500, and 1:1000, respectively, with blocking buffer.

**Protein Estimation**

Protein content was estimated by the Bradford procedure (2) with BSA serving as standard.

**Analysis of Cyanogenic Content**

Prunasin and amygdalin were extracted from developing fruits in methanol, resolved by HPLC, and quantitated by assaying HCN levels after their complete enzymic hydrolysis. A known number of cherries (or selected parts thereof) was weighed, pulverized in liquid N2 in a mortar (precooled by liquid N2), and extracted with 70 mL boiling methanol for 5 min. The sediment was recovered by filtration through glass wool and reextracted with 50 mL boiling 70% methanol for 5 min. Insoluble components were removed by filtration, and the combined filtrates were centrifuged at 17,600 g for 10 min. The supernatant was reduced in volume to 0.5 to 1.0 mL by evaporation overnight under a fume hood followed by rotary evaporation (below 50°C). Cyanoglycosides were quantitatively transferred to 2-mL graduated tubes using 70% (v/v) methanol as solvent, centrifuged in closed Eppendorf tubes in a Beckman Microfuge E for 5 min, and stored at −20°C until analyzed. HPLC was performed using a Beckman 110A pump linked to a Beckman 153 UV detector (254 nm). Cyanoglycosides were resolved by injecting an aliquot (100 μL) onto an UltraspHERE-ODS column (10 × 250 mm, Altex) and eluting isocratically at room temperature with 38% (v/v) methanol at 2.0 mL/min. Average retention times for amygdalin and prunasin were 9.5 and 13.1 min, respectively. Fractions were collected between 6 and 17 min, reduced to dryness by rotary evaporation (under 50°C), and redissolved in 2 mL 0.1 M potassium phosphate buffer, pH 6.8. An aliquot (0.5 mL) was incubated overnight at 37°C in shaking Thunberg tubes with 0.3 mL almond emulsion (containing 7.2 units β-glucosidase activity). Liberated HCN was absorbed in traps...
containing 0.7 mL 1 M NaOH. The traps were rinsed with an additional 0.2 mL 1 M NaOH, and the released HCN was measured by standard methods (1) using standard sodium cyanide solutions for quantitation. When subjected to HPLC and cyanide analysis as described above, recoveries of authentic prunasin and amygdalin routinely exceeded 97%.

After endocarp development began (28 DAF), fruits were dissected into two fractions, namely the developing seeds (contents of the endocarp) and the remaining tissues (the pericarp) for separate analysis.

Presentation of Data

In keeping with earlier studies (7), the potential cyanogenicity of fruits during their maturation has been emphasized here by expressing cyanogen and enzyme levels on a per fruit (or fruit part) basis. To facilitate comparison with immunoblot analysis data, the enzyme levels are also given on a per g fresh weight basis. The complexity of chromatographic techniques rendered unfeasible the processing of multiple samples at each time-point. Nevertheless, each data point represents an average derived from many (n = 8–750) fruits or fruit parts.

RESULTS

Developmental Stages of Fruit Maturation

In common with many other fleshy drupaceous fruits (26, 28), the development of P. serotina fruits showed three distinct growth phases (12). During phase I (0–28 DAF), the fruits exhibited a dramatic increase both in size and in fresh and dry weights (Fig. 1). During mid-phase I, the endocarp began differentiation and by 28 DAF, was quite woody and enclosed glassy-looking nucellar and endosperm tissues (Fig. 2A). Thereafter, a period of retarded growth (phase II; 29–65 DAF) ensued during which the embryo developed within and at the expense of the nucellus and endosperm (Fig. 2, B and C). Cotyledons became visible to the naked eye approximately 42 DAF and, by the end of phase II (65 DAF), they occupied most of the seed volume, whereas the residual endosperm tissue was restricted to two thin strips of cells associated with the testa. Ripening of the mesocarp (m) includes an increase in cell size and accumulation of anthocyanins and sugars.

Figure 2. Anatomy of black cherry fruits during three characteristic phases of development. A, Mid-phase I. Easily visible are the nucellar (n) and endosperm (e) tissues enclosed within the increasingly woody endocarp (w). The embryo is difficult to detect at this stage. B, Mid-phase II. The cotyledons (c) of the rapidly developing embryo are now clearly visible. C, Late phase II. The nucellar and endosperm tissues diminish markedly in size as they supply nutrients for embryo development. D, Phase III. The cotyledons now occupy the majority of the seed volume, whereas the residual endosperm tissue is restricted to two thin strips of cells associated with the testa.

Figure 1. Growth of maturing black cherry fruits from flowering to maturity. Mean fresh weights (●) and dry weights (●) were determined as described in "Materials and Methods."
the cherries finally assumed a black hue (Fig. 2D). Although the lengths of phases I through III are sensitive to climatic conditions and thus vary somewhat from season to season, changes in levels of the cyanogens (and their catabolic enzymes) follow similar trends as those detailed below for the 1990 season.

Cyanogenic Glycoside Levels during Fruit Maturation

The levels of prunasin and amygdalin in whole fruits followed markedly different time-courses during fruit maturation (Fig. 3A). Parallelizing observed changes in fruit weights, prunasin levels rose quickly during phase I and reached approximately 3.1 μmol/cherry by 28 DAF. At this time, the seed and pericarp tissues contained roughly equal amounts of prunasin (Fig. 3, B and C). After attaining a maximum of 3.5 μmol/fruit at 42 DAF, whole fruit prunasin levels declined throughout the remainder of phases II and III (Fig. 3A). In contrast, amygdalin levels rose steeply from near zero at 35 DAF to exceed 5 μmol/fruit by 76 DAF.

Beginning 28 DAF, fruits were also dissected into two fractions, namely the developing seeds and the pericarp, for individual analysis of their cyanoglycoside contents. Seed tissues at 28 DAF, which consisted largely of nucellar and endosperm tissues enclosed within the integuments, possessed 1.36 μmol prunasin/fruit unit but lacked amygdalin (Fig. 3B). Concomitant with cotyledon development (approximately 42 DAF), amygdalin levels increased rapidly and eventually exceeded 3 μmol/seed by 76 DAF. In contrast, prunasin levels declined during cotyledon development and were negligible by 76 DAF. Similar changes in cyanogenic glycoside levels occurred in the pericarp (Fig. 3C). Between 28 and 56 DAF, prunasin levels remained roughly constant at approximately 1.6 μmol/fruit unit before decreasing to 0.5 μmol/unit by 76 DAF. Amygdalin began accumulating in the pericarp 35 to 42 DAF and steadily increased, reaching a plateau concentration of approximately 2 μmol/unit at 76 DAF. At maturity, cyanoglycosides were detectable in both exocarp (epidermis) and mesocarp using the semi-quantitative Feigl-Anger test (6).

Levels of AH, PH, and MDL during Fruit Maturation

The ability of developing black cherry fruits to release significant levels of HCN upon tissue disruption was first noted during mid-phase II, as judged by the Feigl-Anger test (6). Direct enzyme assays provided an explanation for this finding. Coincident with cotyledon development and amygdalin accumulation, the levels of AH, PH, and MDL increased rapidly and specifically within seed tissues (Fig. 4A). Such dramatic increases in enzyme activity might result from (a) enzyme induction, (b) activation of preexisting, inactive enzyme forms, or (c) loss of hypothetical inhibitors that suppressed these activities during phase I. To distinguish between these possibilities, proteins contained within cell-free extracts of maturing seeds (28–81 DAF) were resolved by SDS-PAGE, blotted onto nitrocellulose, and probed using three polyclonal antibody preparations having demonstrated monospecificities for AH, PH, and MDL, respectively. Immunoblotting revealed that these proteins were absent from developing seeds until their coordinate induction during mid-phase II (Fig. 4B). First detected 49 DAF, their levels quickly increased, eliciting strong immunostaining responses until maturity.

In contrast with their high activities in maturing seeds, AH, PH, and MDL were not detectable in cell-free extracts of the pericarp at any developmental stage by either direct enzyme assay or immunoblotting (data not shown). Thus, despite its high cyanoglycoside content during phases I through III (Fig. 3C), the pericarp appears acyanogenic because it lacks the enzymatic machinery to release HCN upon tissue disruption. These data explain why macerated tissues from immature fruits elicited only a weak response in the Feigl-Anger test until exogenous β-glucosidases (almond emulsin) were provided (20). Such behavior is also exhibited by the mesocarp of ripe, commercially marketed peaches (data not shown).

DISCUSSION

The pattern of accumulation of amygdalin and prunasin in developing black cherries was similar to those seen in other rosaceous stone fruits (4, 7, 14). Although prunasin predominates during phase I, the synthesis of amygdalin during later
phases, concomitant with a decline in prunasin levels, causes the disaccharide to predominate (>85%) at maturity. By assaying the levels of AH, PH, and MDL in various fruit parts, the current study reveals their tissue localization with respect to those of the glycosidic substrates. It also identifies the developmental stages when fruits have the ability to release HCN.

Until mid-phase II, fruits are acyanogenic because they contain cyanoglycosides (mostly prunasin) but lack the catabolic enzymes. Thereafter, the developing *P. serotina* seeds accumulate both components of the so-called "cyanide bomb" and are thus potentially highly cyanogenic. The mode of cellular or subcellular compartmentation within uncrushed seeds that prevents the premature, suicidal degradation of the cyanoglycosides is currently under investigation in our laboratory using immunocytochemical techniques (29). Upon tissue disruption, the glycosides are rapidly degraded by AH, PH, and MDL releasing HCN and benzaldehyde (10, 11, 30). The documented toxicity of *P. serotina* toward livestock and humans supports the notion that these reaction products serve to defend the propagules against herbivory (9). Whether amygdalin also serves as a nitrogen source for germinating seedlings remains to be evaluated.

The flesh of rosaceous stone fruits such as peaches and apricots is generally regarded as acyanogenic. Likewise, the black cherry pericarp is also acyanogenic. Although it accumulates high levels of cyanogenic glycosides throughout fruit maturation, it lacks the enzymes AH, PH, and MDL that would release HCN. This situation is not unique to *Prunus* species. For example, few Australian *Acacia* species actually possess the β-glucosidases required to hydrolyze endogenous cyanogenic glycosides (23). The physiological role(s) played by the *P. serotina* pericarp glycosides is (are) unclear. It is possible that the unhydrolyzed cyanoglycosides might contribute toward defense through their bitterness, as was shown for the glycoside cardiospermin (3). Certainly, immature fruits suffer little predation until late-phase III, when the fruits soften, change color, and become attractive to birds, notably robins. At this time, the bitterness of the glycosides might be offset by sugar accumulation. Pericarp cyanoglycosides could also contribute to defense against herbivores through release of HCN and benzaldehyde if fruits were sufficiently damaged as to allow mixing of macerated pericarp tissues with the seed β-glucosidases.

ACKNOWLEDGMENTS

The authors wish to thank Mark Woerner for photographic reproductions and Linda Donohoe and Tony Nevshemal for assistance in manuscript preparation.

LITERATURE CITED


Figure 4. Levels of AH, PH, and MDL in developing *P. serotina* seeds. A. Extracts of developing seeds (*n* = 8–750, depending on developmental stage) were partially purified as described in "Materials and Methods." Enzyme activities are expressed on a per fruit (O) or a per g fresh weight (●) basis. Each data point represents the mean of three replicates whose variability did not exceed the dimensions of the symbol shown. B. Immunoblot analysis of AH, PH, and MDL levels. Equal volumes (30 μL) of crude seed homogenates, derived from maturing seeds as described in "Material and Methods," were subjected to SDS-PAGE and immunoblot analysis using polyclonal antibodies monospecific for each protein.


