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Cyanogenesis in glucosinolate-producing plants: *Carica papaya* and *Carica quercifolia*☆

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Abstract

(*R*)-2-(β-D-Glucopyranosyloxy)-2-phenylacetonitrile (prunasin) was isolated from *Carica papaya* L. and *C. quercifolia* (A. St.-Hil.) Hieron. (syn. *C. hastata* Brign.). Earlier reported presence of cyclopentanoid cyanohydrin glycosides in *C. papaya* could not be confirmed, and no cyclopentanoid amino acids could be detected in extracts of *C. papaya* and *C. quercifolia*. Conversion of [2,3,4,5,6-^{3}H]phenylalanine into tritiated prunasin was demonstrated in both species. On the other hand, when the plants were administered [2-^{14}C]-2-(2′cyclopentenyl)glycine, extracted, and the extracts hydrolyzed with β-glucosidase (*Helix pomatia*), formation of labelled cyanide was not observed. The absence of cyclopentanoids, which are typical for the Passifloraceae, and the inability of *Carica* species to utilize 2-(2′-cyclopentenyl)glycine as a precursor of cyanogenic glycosides are in agreement with the relative phylogenetic position of the Caricaceae and the Passifloraceae. *Carica* species are thus rare examples of taxa in which glucosinolates and cyanogenic glycosides co-occur, both types of natural products being derived from the same amino acid, phenylalanine.

Keywords: *Carica papaya*; *Carica quercifolia*, *Carica hastata*; Caricaceae; Passifloraceae; Biosynthesis; Glucosinolates; Cyanohydrin glycosides; Cyanogenic glycosides; Prunasin; Tetraphyllin B; Cyclopentenylglycine.

1. Introduction

During the recent years, outstanding advances have been made in understanding of the biosynthesis of cyanogenic glycosides (Jones et al., 1999; Møller and Seigler, 1999) and glucosinolates (mustard oil glucosides) (Du et al., 1995; Bak et al., 1998; Hull et al., 2000; Mikkelsen et al., 2000; Wittstock and Halkier, 2000; Hansen et al., 2001; Kroymann et al., 2001; Reintanz et al., 2001). Cytochrome P450 enzymes (CYP79 family) are involved in conversion of amino acids to oximes, the latter being the branching point separating biosynthetic pathways to the two classes of plant metabolites. The amino acid sequence and function of oxime-metabolizing cytochromes are of considerable interest from the evolutionary point of view, as well as because of the possibility of engineering of the biosynthesis.

In general, plants producing glucosinolates are not cyanogenic, and cyanogenic plants do not produce glucosinolates. A notable exception was discovered in 1984. Thus, *Carica papaya* L. (pawpaw), long-known to produce benzylglucosinolate (**1**) (Ettlinger and Hodgkins, 1956), was reported to contain a cyclopentanoid cyanogenic glycoside, tetraphyllin B (**2**), along with prunasin (**3**) (Spencer and Seigler, 1984). More recently, the cyanogenesis of *C. papaya* was confirmed (Bennett et al., 1997). However, the biosynthetic origin of the cyanide produced by various tissues of the plant and the identity of cyanogenic glycoside (or glycosides) present were not investigated. Because of the interesting ability of this plant to accumulate two types of aldoxime metabolites, a reinvestigation of the cyanogenesis of *C. papaya* was performed. *C. quercifolia* was included in the study in order to determine whether the chemistry of *C. papaya* is representative for the genus.

2. Results and discussion

The classical methods of detection of cyanohydrin glycosides in plants, such as autolysis in a closed vial

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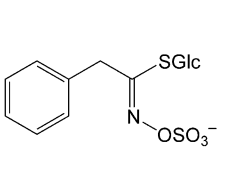

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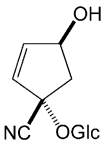

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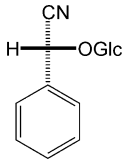

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and a color reaction specific for the liberated hydrogen cyanide (Feigl and Anger, 1966; Brimer, 1994) failed to detect cyanogenesis of *C. papaya* and *C. quercifolia*. This could be due either to a limited sensitivity of the cyanide detection method or to the absence of endogenous β-glucosidase. However, extraction of the plant material followed by the cyanide specific, TLC sandwich picrate assay (Brimer et al., 1983) using concentrated fractions readily demonstrated the presence of a cyanogenic constituent.

Preparative-scale extraction of *C. papaya* leaves, followed by fractionation by silica gel chromatography and finally by preparative reversed-phase HPLC, resulted in isolation of a small amount (0.005% of dry wt) of prunasin (**3**). The same product was found in leaves from several different specimens, and was also isolated from fruits, stems, flowers and roots, but no cyanogenic material could be detected in seeds. Leaves of *C. quercifolia* also yielded prunasin (**3**). In all cases prunasin was isolated as the only cyanogenic constituent, in spite of an extensive search using the TLC sandwich method, normal-phase and reversed-phase HPLC, as well as 500 MHz ^{1}H NMR. No material eluting as authentic tetraphyllin B (**2**) (Jaroszewski and Olafsdottir, 1986) was observed in HPLC, and no cyanogenic spot corresponding to **2** could be identified by TLC. Investigation of chromatographic fractions by ^{1}H NMR failed to detect the characteristic resonances of the olefinic protons H-2 and H-3, or of the diastereotopic protons H-5A and H-5B of **2** or of other cyclopentanoid cyanogenic glycosides (Jaroszewski and Jensen, 1985; Jaroszewski and Olafsdottir, 1986; Jaroszewski et al., 1987, 2002). Prunasin (**3**) isolated from *C. papaya* and *C. quercifolia*, characterized by ^{1}H and ^{13}C NMR spectra of the free glycoside and of its tetraacetate, was pure and was not accompanied by passiedulin or benzyl β-D-glucopyranoside, recently detected in *Passiflora edulis* (Christensen and Jaroszewski, 2001). Amino acid fraction of *C. papaya* prepared by ion exchange chromatography and analyzed by ^{1}H NMR as recently described (Andersen et al., 2001; Clausen et al., 2001, 2002; Wellendorph et al., 2001) did not contain any detectable amounts of 2-(2′-cyclopentenyl)glycine, the amino acid precursor of cyclopentanoid cyanogenic glycosides (Tober and Conn, 1985; Olafsdottir et al., 1992; Jaroszewski et al., 1996). The method used is capable of detecting at least 0.0002% of 2-(2′-cyclopentenyl)glycine (Clausen et al., 2002).

When the leaves of *C. papaya* and *C. quercifolia* were fed with L-[2,3,4,5,6-^{3}H]phenylalanine, the plant material extracted, cold prunasin added to the extract as a carrier, and prunasin isolated by solid-phase extraction and purification by preparative HPLC, significant incorporation of the radioactivity into the cyanogenic glycoside (0.10–0.63%) was observed in both plants. The observed level of incorporation was as typically observed in the biosynthesis of cyanohydrin glycosides from amino acids in intact plant tissue (Olafsdottir et al., 1992). This is the first confirmation of the biosynthesis of **3** from phenylalanine in the Caricaceae.

In another experiment, (2*RS*,1′*RS*)-[2-^{14}C]-2-(2′-cyclopentenyl)glycine was administered to the leaves of *C. papaya* and *C. quercifolia* and the crude extract subjected to hydrolysis with *Helix pomatia* enzymes (which include β-glucosidase), trapping the released hydrocyanic acid in an aqueous solution of sodium hydroxide by the method previously described (Olafsdottir et al., 1992). The cyanide formed from the labelled extract was not radioactive, as it would be expected in the case of formation of **2**, which includes incorporation of the specifically labelled C-2 of the amino acid into the cyano group. This demonstrates the inability of *C. papaya* and *C. quercifolia* to convert 2-(2′-cyclopentenyl)glycine to a cyanogenic glycoside.

The cyanogenesis of *C. papaya* and the accumulation of prunasin could thus be confirmed in the present work (Jaroszewski, unpublished results cited in Hegnauer, 1989), but there is no evidence for the presence of **2** or biosynthesis of a cyclopentanoid cyanogenic glycoside from 2-(2′-cyclopentenyl)glycine. Identical results were obtained with *C. quercifolia*, the cyanogenesis of which was not investigated prior to this work. Both *C. papaya* (Ettlinger and Hodgkins, 1956) and *C. quercifolia* (Gmelin and Kjær, 1970) produce benzylglucosinolate (**1**). Thus, cyanogenic glycosides and glucosinolates in the Caricaceae originate from the same precursor amino acid.

In the four major taxonomic systems (Thorne, 1992; Dahlgren, 1989; Cronquist, 1988; Takhtajan, 1986) the Caricaceae are placed in the Violales together with a group of plants including Passifloraceae, which produce cyclopentanoid cyanogenic glycosides (Jaroszewski et al., 2002). The alleged presence of **2** in *C. papaya* suggested that the Caricaceae are intermediate in chemistry between the Passifloraceae and the Capparales (Spencer and Seigler, 1984). The present results show that this link between the Caricaceae and the Passifloraceae is not real (Hegnauer, 1989; Jørgensen, 1995). Caricaceae are a typical glucosinolate-producing family containing stomatal myrosin cells (Jørgensen, 1995), which are absent in the Passifloraceae. Analysis of the *rbc*L gene sequences by Rodman and coworkers demonstrated that the Caricaceae belong to the main glucosinolate-containing clade also including the Brassicaceae, Capparaceae, Tovariaceae, Resedaceae, Gyrostemonaceae,

Bataceae, Limnanthaceae, Moringaceae, Tropaeolaceae, Bretschneideraceae, Akaniaceae, Salvadoraceae and Pentadiplandraceae, and that Caricaceae are apparently most close to the Moringaceae (Rodman et al., 1992, 1993, 1994, 1996; Gadek et al., 1992). DNA sequencing of the nuclear 18S ribosomal RNA gene has yielded the same result (Rodman et al., 1998). Recent phylogenetic analyses of flowering plants based on gene sequencing have resulted in a new classification at the ordinal level (APG, 1998; Savolainen et al., 2000). Here the order Capparales (Brassicales) belongs to the higher category eurosids II, while the Passifloraceae and related families producing cyclopentanoid cyanogenic glycosides (e.g. Turneraceae and Kiggelariaceae) together with the Violaceae belong to the order Malphigiales within the eurosids I.

We expect that *Carica* species, because of their ability to synthesize a cyanohydrin glycoside and a glucosinolate in parallel, will prove to be a valuable object for studies of genetics, regulation and evolution of the two biosynthetic pathways involved. It seems likely that *Carica* has only a single benzaldehyde oxime-forming cytochrome P450 at the biosynthetic branching point.

3. Experimental

3.1. General

NMR spectra were recorded on a Bruker AM5000 or AM250 spectrometer, using tetramethylsilane as an internal standard. Column chromatography was performed using open columns packed with Merck silica gel 60 (66–200 μm). Preparative HPLC separations were performed on Lichrosorb Si 60 (7 μm, 16×25 cm column) or Lichrosorb RP-18 (7 μm, 1.6×25 cm column) with refractive index and spectrophotometric detection. Cyanogenic compounds were detected by TLC on 20×20 cm Merck precoated aluminum plates (silica gel 60 F_{254}) run with EtOAc–Me_2CO–CH_2Cl_2–MeOH–H_2O (20:15:6:5:4), using the cyanide-specific sandwich picrate assay (Brimer et al., 1983). *Helix pomatia* enzyme preparation (β-glucuronidase, crude enzyme) and prunasin were obtained from Sigma Chemical Co. L-[2,3,4,5,6-^{3}H]Phenylalanine was purchased from Amersham Bioscience. (2*RS*,1′*RS*)-[2-^{14}C]-2-(2′-cyclopentenyl)glycine was synthesized as previously described (Olafsdottir et al., 1992). Isolute C18(EC) SPE cartridges from International Sorbent Technology, Ltd., containing 1 g of the sorbent, were used for solid phase extraction.

3.2. Plant material

Two specimens of *Carica papaya* L. (voucher DFHJJ22 and DFHJJ23) and one specimen of *C. quercifolia* (A. St.-Hil.) Hieron. syn. *C. hastata* Brign. (voucher DFHJJ24) were grown in a greenhouse of the Botanic Garden, University of Copenhagen, Copenhagen. Another specimen of *C. papaya* L. (voucher DFHJJ25) was cultivated outdoors in McAllen, TX, USA. Voucher specimens were deposited in Herbarium C (Botanical Museum, University of Copenhagen, Copenhagen).

3.3. Isolation of prunasin (*3*) from C. papaya

Fresh leaves of *C. papaya* (DFHJJ22) were frozen with liquid nitrogen, freeze-dried, and finely pulverized. The powder (230 g) was extracted twice with boiling MeOH–H_2O (4:1), the extracts evaporated, the residue adsorbed on silica gel, and chromatographed on a 8×35 cm column of silica gel with EtOAc–Me_2CO–CH_2Cl_2–MeOH–H_2O (20:15:6:5:4), collecting 25-ml fractions. The fractions were monitored by TLC (silica gel, same solvent) using a sugar-specific (naphthoresorcinol reagent) as well as a cyanide-specific (sandwich picrate assay) method of visualizing the spots. Since the amount of cyanogenic material expected was low, the fractions were concentrated by evaporation prior to TLC. Only one cyanogenic band was observed, with R_f value of 0.41 corresponding to authentic prunasin. The appropriate fractions were combined, and chromatographed again on a 25×70 cm column of silica gel, using EtOAc–MeOH–H_2O (95:5:2). The cyanogenic material was further purified by preparative HPLC in Lichrosorb Si60 using 5 ml/min of EtOAc–MeOH–H_2O (93:5:2). The yield was 10.8 mg (0.005% dry wt) of crude prunasin (**3**), finally purified on the latter column with EtOAc–MeOH–H_2O (85:13:2). ^{1}H NMR (250 MHz, CD_3OD) δ 7.60 (*m*, 2H, aromatic), 7.46 (*m*, 3H, aromatic), 5.91 (*s*, 1H, cyanohydrin), 4.23 (*d*, J=7.5 Hz, 1H, H-1′), 3.92 (*dd*, J=12.0 and 2.2 Hz, 1H, H6′B), 3.70 (*dd*, J=12.0 and 5.8 Hz, 1H, H6′A), 3.23–3.32 (*m*, remaining glucosyl protons); the spectrum was identical with that of authentic prunasin. ^{13}C NMR (62.9 MHz, CD_3OD): δ 134.9 131.1, 130.1, and 129.0 (aromatic), 119.5 (CN), 101.9, 78.4, 77.9, 74.8, 71.6 and 62.8 (glucopyranosyl), 68.4 (cyanohydrin); the spectrum was identical with that of authentic prunasin.

For further confirmation of the identity, the sample was acetylated (overnight treatment with Ac_2O–pyridine at room temp.), and the product analyzed by ^{1}H and ^{13}C NMR. ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.45 (*br s*, 5H, aromatic), 5.52 (*s*, 1H, cyanohydrin), 5.05–5.15 (*m*, 4H, H2′-H4′), 4.53 (*d*, J=7.5 Hz, 1H, anomeric), 4.25 (*dd*, J=12.2 and 5.0 Hz, 1H, H6′B), 4.13 (*dd*, J=12.2 and 2.5 Hz, 1H, H6′A), 3.64 (*m*, 1H, H5′), 2.12 (*s*, 3H, acetyl), 2.01 (*s*, 3H, acetyl), 2.00 (*s*, 6H, two acetyls), the spectrum was identical with that of a sample prepared from authentic prunasin. ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 170.8, 170.2, 169.3 and 169.0 (carbonyl), 132.1, 130.5, 129,3, and 127.7 (aromatic), 116.7

(nitrile), 98.3, 72.6, 72.3, 70.9, 68.6, 68.1 and 61.7 (glycopyranosyl and cyanohydrin), 20.5–20.7 (acetyls); the spectrum was identical with that of a sample prepared from authentic prunasin.

HPLC investigation of various fractions from the silica gel column, using LiChrosorb RP-18 column with 5 ml/min of MeOH–H_2O (1:2) failed to detect material with retention time of tetraphyllin B (**2**) (R_t 16.5 min).

Extraction of leaves from other specimens of *C. papaya* (DFHJJ23 and DFHJJ25) also yielded prunasin as the only cyanogenic constituent. In addition to the leaves, other parts of *C. papaya* (DFHJJ23) were investigated: stem (41 g dry weight), root (50 g), unripe fruit (60 g), seeds (10 g), flowers (21 g). Extraction and fractionation as above by CC and HPLC yielded prunasin as the only cyanogenic constituent (identified by ^{1}H NMR as described above) from all plant parts except for the seeds, where no cyanogenic material could be detected.

3.4. Isolation of prunasin (*3*) from C. quercifolia

Extraction of 93 g of dry leaves of *C. quercifolia* (DFHJJ24) and fractionation of the extract similarly as described above for *C. papaya*, yielded 4 mg (0.004%) of prunasin (**3**), identified as above.

3.5. Tracer experiments

Leaves of *C. papaya* (DFHJJ22) or *C. quercifolia* (DFHJJ24) weighting 5–8 g were administered radioactive amino acids by immersing stalks in 4 ml of H_2O containing 5.0 μCi of L-[2,3,4,5,6-^{3}H]phenylalanine or 5 μCi of [2-^{14}C]-2-(2′-cyclopentenyl)glycine. Each feeding experiment was performed in duplicate. After 36 h metabolic period (12 h with artificial sunlight, 12 h in dark, 12 h with artificial sunlight) the plant material was ground in liquid nitrogen and extracted with boiling MeOH–H_2O (4:1), and the extracts evaporated in vacuo.

Extracts of plants fed with L-[2,3,4,5,6-^{3}H]phenylalanine were dissolved in 10 ml of H_2O, 5.0 mg of prunasin (**3**) was added to each solution, and the solutions passed through Isolute SPE columns. The columns were rinsed with water and then eluted with 10% MeOH, 20% MeOH and 60% MeOH, and the extracts evaporated. Incorporation of radioactivity into prunasin (**3**), present in 60% MeOH eluates (TLC, ^{1}H NMR), was determined by liquid scintillation counting after purification by preparative HPLC on LiChrosorb RP-18 with 5 ml/min of MeOH–H_2O (1:2). The incorporation of radioactivity into prunasin was as follows: *C. papaya* 0.34 and 0.63%; *C. quercifolia* 0.31 and 0.10% (duplicate feeding experiments).

Extracts of plants fed with [2-^{14}C]-2-(2′-cyclopentenyl)glycine were dissolved in 3 ml of water, and 2.5 of each solution placed in the outer chamber of Conway microdiffusion cells. The inner chamber of the cells contained 3.0 ml of 0.5 M aq. NaOH. After addition of 0.5 ml of the *Helix pomatia* enzyme preparation to the outer chamber the hydrolysis was continued for 48 h, after which the amount of radioactivity in the 0.5 M NaOH solution in the inner chamber, which contained trapped HCN, was determined. No radioactivity transfer to the NaOH solution was observed.

3.6. ^{1}H NMR analysis of amino acid fraction from C. papaya

Freeze-dried leaves of *C. papaya* (26 g) were extracted with boiling MeOH–H_2O (4:1) and amino acids with polarity corresponding to cyclopentenylglycines were isolated by ion-exchange on Dowex-50W followed by silica gel chromatography, essentially according to the procedure previosly described (Clausen et al., 2002). The amino acid fraction was analyzed for cyclopentenylglycines by ^{1}H NMR in D_2O at pD = 6.5 (Clausen et al., 2002). No characteristic resonances at δ 5.5–6.2 were present.

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