Catabolism of (*R*)-Amygdalin and (*R*)-Vicianin by Partially Purified β-Glycosidases from *Prunus serotina* Ehrh. and *Davallia trichomanoides*

Gary Kuroki, Pauline A. Lizotte, and Jonathan E. Poulton
Department of Botany, University of Iowa, Iowa City, Iowa 52242

Z. Naturforsch. **39 c**, 232–239 (1984); received October 3, 1983

Cyanogenic Disaccharides, β-Glycosidases, *Prunus serotina*, *Davallia trichomanoides*, Amygdalin, Vicianin

Mature black cherry (*Prunus serotina* Ehrh.) seeds accumulate high levels of the cyanogenic disaccharide (*R*)-amygdalin. Extracts from these seeds contain two β-glycosidases which have been identified and completely resolved by DEAE-cellulose ion-exchange chromatography. Amygdalin hydrolase hydrolyzed (*R*)-amygdalin at an optimum pH of 5.5, releasing (*R*)-prunasin and D-glucose. This enzyme showed highest activity towards (*R*)-amygdalin and failed to hydrolyze (*R*)-prunasin, linamarin, β-gentiobiose and cellobiose. A distinct β-glycosidase, prunasin hydrolase, displayed a pronounced preference for (*R*)-prunasin, hydrolyzing this cyanogenic monosaccharide at an optimum pH of 6.5 to mandelonitrile and D-glucose. Prunasin hydrolase was inactive towards (*R*)-amygdalin, linamarin, and β-gentiobiose. Both enzymes showed significant activity towards the artificial substrates β-ONPGlu and β-PNPGlu but did not hydrolyze α-PNPGlu. In view of the pronounced specificity of these enzymes towards endogenous cyanogens, it is concluded that upon disruption of black cherry seeds (*R*)-amygdalin is catabolized to mandelonitrile in a stepwise manner (the sequential mechanism) by amygdalin hydrolase and prunasin hydrolase with (*R*)-prunasin serving as intermediate.

Young fronds of *Davallia trichomanoides* are rich sources of (*R*)-vicianin (the β-vicianoside of (*R*)-mandelonitrile). A β-glycosidase, vicianin hydrolase, has been partially purified from frond extracts by ion-exchange chromatography. At the optimum pH of 6.0, this enzyme showed highest hydrolytic activity with (*R*)-vicianin, although both (*R*)-amygdalin and (*R*)-prunasin could be hydrolyzed at approximately 15% of the rate observed with (*R*)-vicianin. It failed to hydrolyze β-gentiobiose, cellobiose, linamarin and α-PNPGlu. Closer examination revealed that (*R*)-vicianin and (*R*)-amygdalin were hydrolyzed at the aglycone-disaccharide bond (the simultaneous mechanism) yielding mandelonitrile and the respective disaccharides vicianose and β-gentiobiose.

Introduction

The catabolism of cyanogenic glycosides is initiated by the cleavage of the carbohydrate moiety by one or more β-glycosidases [1]. Although the catabolism of several cyanogenic monosaccharides is now well understood [2], the mode by which cyanogenic disaccharides are hydrolyzed remains largely unclear. Theoretically, two distinct degradative pathways may exist for cyanogenic disaccharides, as shown in Fig. 1. Hydrolysis at the aglycone-disaccharide bond would release the α-hydroxynitrile and a disaccharide (the "simultaneous" mechanism). Alternatively, the two sugar residues might be removed singly by stepwise hydrolysis with a cyanogenic monosaccharide acting as intermediate (the "sequential" mechanism). In the latter case, the two hydrolytic steps might be catalyzed by the same or by distinct β-glycosidases. Previous reports in the literature [3–6] suggest that both simultaneous and sequential pathways may exist in Nature. In order to provide more information about this subject, we have selected two cyanogenic plant tissues for closer examination, namely, *Prunus serotina* seeds and *Davallia trichomanoides* fronds. These tissues accumulate high concentrations of the cyanogenic disaccharides (*R*)-amygdalin (the β-gentiobioside of (*R*)-mandelonitrile) and (*R*)-vicianin (the β-vicianoside of (*R*)-mandelonitrile), respectively. Cyanogen-specific β-glycosidases have been partially purified from

Abbreviations: PVP, polyvinylpolypyrrolidone; AH, amygdalin hydrolase; PH, prunasin hydrolase; VH, vicianin hydrolase; β-PNPGlu and β-ONPGlu, *para*- and *ortho*-nitrophenyl-β-D-glucopyranosides; β-PNPXyl and β-ONPXyl, *para*- and *ortho*-nitrophenyl-β-D-xylopyranosides; β-PNPGal, *p*-nitrophenyl-β-D-galactopyranoside; α-PNPGlu, *p*-nitrophenyl-α-D-glucopyranoside.

Reprint requests to Dr. J. E. Poulton.

0341-0382/84/0300-0232 $ 01.30/0

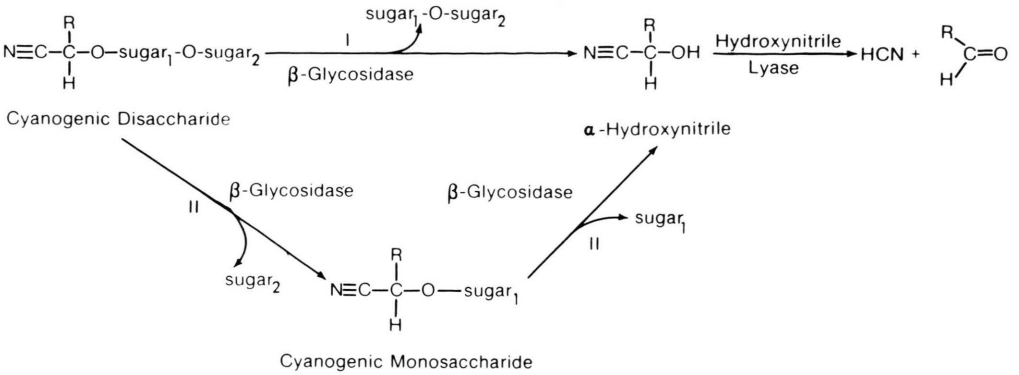

Fig. 1. The simultaneous (I) and sequential (II) mechanisms for catabolism of cyanogenic disaccharides.

these plants and their behavior towards endogenous cyanogens and other glycosidic substrates investigated. This has allowed us to predict the pathway by which the endogenous cyanogenic disaccharides are catabolized upon tissue disruption.

Materials and Methods

Chemicals

Polyvinylpolypyrrolidone (PVP), β-glucosidase (type II, from almonds), simple sugars and chromogenic substrates were purchased from Sigma Chemical Co., St. Louis, MO. Glycosidic substrates were available from our laboratory collection. Silica gel K5 TLC plates (thickness, 250 μ), 3MM chromatography paper and DEAE-cellulose were obtained from Whatman Chemical Separation Ltd., Kent, U.K., Microcrystalline cellulose (Avicel) was purchased from Merck, Darmstadt, BRD. Vicianin was obtained by grinding *Vicia angustifolia* seeds in liquid nitrogen with a mortar and pestle. The powdered seeds were placed in boiling 70% methanol for 10 min and filtered through Whatman grade 1 filter paper. The filtrate was extracted with an equal volume of petroleum ether. The methanolic fraction was evaporated to dryness, redissolved in water, and applied to Whatman 3MM chromatography paper. The paper was developed in *n*-butanol:acetic acid:H_2O (4:1:5, by vol., upper phase). After thoroughly drying the paper, the cyanogen was located by the Feigl-Anger "sandwich" technique [7] employing the *Davallia* β-glycosidase preparation to release HCN. The glycoside was eluted with methanol and applied to a cellulose column (1.0 × 41 cm). The column was eluted with water-saturated *n*-butanol and fractions containing vicianin were identified by the Feigl-Anger test [8]. Vicianin was recrystallized from benzene:methanol (1:1, by vol.) and its purity was confirmed by thin layer chromatography. Clorox was purchased from the Clorox Co., Oakland, Ca.

Plant materials

Mature black cherry (*Prunus serotina* Ehrh.) fruits were collected from Hickory Hill Park, Iowa City. The pits were removed and surface sterilized in 10% Clorox, blotted dry and stored at 4 °C until used. *Davallia trichomanoides* specimens were purchased from Fountain Square Nurseries, Sacramento, Ca.

Purification and assay of amygdalin hydrolase and prunasin hydrolase from Prunus serotina

Unless otherwise indicated, all stages were carried out at 4 °C. Black cherry pits (approximately 35) were cut in half with a razor blade and the seeds homogenized with 10 ml of buffer I, 0.5 g PVP, and 2.0 g glass beads in a mortar. The homogenate was filtered through four layers of cheesecloth and centrifuged at 17600 × *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 this buffer. The eluate was applied to a DEAE-cellulose column (1.6 × 10 cm) which had been pre-equilibrated with buffer II. After washing the column excessively with buffer II, elution of bound proteins was accomplished with a 0–300 mM NaCl gradient in buffer II. Fractions containing amygdalin hydrolase (AH) and prunasin

hydrolase (PH) activity were pooled separately and dialyzed against buffer II overnight. These enzyme preparations were stored at 4 °C and used for assay of β-glucosidase activity as described below.

The standard assay mixture for AH and PH activity contained 3.75 μmol glycosidic substrate (dissolved in 0.15 ml H_2O), 50 μmol citrate-phosphate buffer (pH 5.5 for AH, and pH 6.5 for PH), 30 μl H_2O, and 20 μl of diluted enzyme preparation (diluted 1:7 with buffer II) in a total volume of 0.25 ml. After incubation at 30 °C for 5 min, the reaction was terminated by transferring an aliquot (0.23 ml) to a test tube immersed in boiling water. After boiling the mixture for 30 s, it was placed on ice. Aliquots (0.2 ml) were removed and glucose production measured by the glucose oxidase procedure [9]. The assay mixture for chromogenic substrates was essentially the same, but the reaction was terminated by adding 2.5 ml of 0.2 M sodium borate-NaOH buffer, pH 9.8. The developed colors were measured at 400 nm.

Purification and assay of vicianin hydrolase from Davallia trichomanoides

All stages were undertaken at 4 °C. Young fronds and fiddle-heads (5 g) of *Davallia trichomanoides* were washed with double-distilled water, blotted gently dry, and homogenized in 30–40 ml of buffer III, 4 g PVP, and 5.0 g of glass beads in a mortar. The homogenate was filtered through four layers of cheesecloth and centrifuged at 17600 × *g* for 20 min. The supernatant liquid was dialyzed against buffer III overnight. The enzyme preparation was then applied to a DEAE-cellulose column (1.6 × 10.5 cm) which had been pre-equilibrated with buffer III. After washing the column with buffer III to remove unbound proteins, elution was accomplished by a linear 0–200 mM NaCl gradient in buffer III. Fractions containing VH activity, which eluted between 70 and 120 mM NaCl, were pooled and concentrated by ultrafiltration. The enzyme was then applied to a Sephadex G-25 column (1.5 × 8.3 cm) which had been pre-equilibrated with buffer IV. Elution was undertaken with buffer IV, and β-glucosidase fractions were stored at 4 °C until assayed for activity by the following methods.

Three assay procedures were employed to determine the activity of vicianin hydrolase towards the various potential substrates. For chromogenic substrates, the enzyme (0.05 ml) was incubated with 10 μmol chromogenic substrate (dissolved in 0.5 ml H_2O) and 0.2 μmol sodium acetate-HCl buffer, pH 6.0, in a total volume of 1.0 ml. After incubation at 30 °C for 1 h, the reaction was terminated by adding 2.0 ml of 0.2 M sodium borate-NaOH buffer, pH 9.8, and the developed colors were measured at 400 nm. For other substrates, the standard assay mixture for β-glucosidase activity contained 0.5 μmol glycosidic substrate (dissolved in 20 μl H_2O), 10 μmol sodium acetate-HCl buffer, pH 6.0, and 20 μl of enzyme in a total volume of 50 μl. After incubation at 30 °C for 1 h, the reaction was terminated by placing the reaction vial in boiling water for 2 min and then on ice. After centrifugation, aliquots (40 μl) were removed and glucose production determined by the glucose oxidase procedure [9].

Since the hydrolysis of vicianin and amygdalin by vicianin hydrolase did not yield glucose (see Results), the above method was ineffective. Hydrolytic rates for these substrates were therefore determined in the following manner. The enzyme preparation (5–40 μl) was incubated at 30 °C with 1 μmol of glycosidic substrate (dissolved in 40 μl H_2O), 10 μmol imidazole-HCl buffer, pH 6.0, and 5 μmol of sodium acetate-HCl buffer, pH 6.0, in a total volume of 0.1 ml. At timed intervals, aliquots (20 μl) were removed and subjected to HPLC (Beckman Model 332 Gradient System) on a reverse-phase Ultrasphere ODS column (4.6 mm × 25 cm) using the solvent 30% methanol at a flow rate of 1.5 ml/min. Hydrolytic rates were calculated from the observed linear decreases in substrate area (determined by an Hewlett-Packard 3390A integrator) with time.

Reaction products of vicianin and amygdalin hydrolysis were also co-chromatographed with authentic samples of amygdalin, prunasin, β-gentiobiose, D-glucose and L-arabinose on a Whatman TLC silica gel K5 plate. The plate was irrigated four times using acetonitrile:H_2O (85:15, by vol.) as solvent [8]. Compounds were detected by spraying the plate with 5% sulfuric acid in methanol before heating it at 110 °C for 30 min. Composition of the disaccharides produced by VH activity was confirmed by subjecting the sugars to hydrolysis in 10% trifluoroacetic acid for 1 h at 100 °C. The products were analyzed by the above TLC procedure.

Buffer solutions

The following buffer solutions were used: (I) 0.1 M sodium acetate-HCl buffer, pH 6.0; (II) 10 mM histidine-HCl buffer, pH 6.0; (III) 50 mM imidazole-HCl buffer, pH 6.0; (IV) 25 mM imidazole-HCl buffer, pH 6.0.

Results and Discussion

β-Glycosidases play important roles in the degradation [2] and biosynthesis [11] of many plant products. Largely through the widespread use of synthetic rather than endogenous substrates during their purification and characterization, these enzymes have in past years been regarded as lacking aglycone specificity [12]. This viewpoint has been recently challenged by the demonstration of several β-glycosidases of secondary metabolism having high specificity for their aglycones [12]. For example, two β-glucosidases isolated from *Triglochin maritima* and *Alocasia macrorrhiza*, plants containing the cyanogenic monosaccharide triglochinin, exhibited high specificity for this substrate [14, 15]. Other cyanogenic glycosides were hydrolyzed very poorly or not at all by these enzymes. Likewise, β-glycosidases purified from *Sorghum bicolor* [16], *Linum usitatissimum* [17], and *Triglochin maritima* [14] showed great specificity towards the endogenous cyanogens dhurrin, linamarin and taxiphyllin, respectively.

Whereas the manner by which cyanogenic monosaccharides are hydrolyzed is becoming clearer [2], no general pattern has yet emerged for the catabolism of the five known cyanogenic disaccharides (*i.e.* amygdalin, vicianin, lucumin, linustatin and neolinustatin) [3–6]. In view of the pronounced specificity of cyanogenic monosaccharidases, it could be reasonably expected that tissues containing cyanogenic disaccharides should also possess β-glycosidases showing specificity for such compounds. As described in the introduction, these compounds could be hydrolyzed by either the simultaneous or sequential routes. These interesting questions have been addressed here for the catabolism of amygdalin and vicianin.

The catabolism of amygdalin in Rosaceous species

The mechanism of amygdalin hydrolysis in cyanogenic plants has been investigated using almonds, almond emulsin and apricots as sources of β-glycosidases. In sharp contrast to the early conclusions of Weidenhagen [18], Haisman and Knight [3] reported kinetic data suggesting that almond emulsin contained two (rather than only one) β-glucosidases involved in amygdalin hydrolysis. Each enzyme was believed to be specific for one hydrolytic stage. Thus, "amygdalin hydrolase" (AH) cleaved amygdalin to prunasin and D-glucose, while "prunasin hydrolase" (PH) hydrolyzed prunasin further to mandelonitrile and D-glucose. These enzymes were later identified by continuous electrophoresis in a free buffer film [19], but, since their complete resolution and purification to homogeneity were not achieved, their kinetic and molecular properties remain unknown. In 1974, Lalégerie [20] purified two β-glucosidase isozymes from sweet almonds. The non-physiological *p*-nitrophenyl-β-D-glucoside (β-PNPGlu) was used as substrate during the purification. These isozymes showed major differences when compared with those of Haisman and Knight. Neither protein displayed absolute substrate specificity towards either amygdalin or prunasin since each was active towards both substrates. Furthermore, the observed K_m values of these cyanogens were 4–5 fold higher than previously reported [3]. In other laboratories, almond emulsin was found to contain three [21] or at least four [22] β-glucosidase isozymes. However, in these studies too, the authors used artificial rather than natural substrates to monitor their enzyme purifications. Thus, Helferich and Kleinschmidt [21] and Grover *et al.* [22] in effect achieved the purification of β-PNPGlu- and salicin-hydrolyzing isozymes but paid no attention whether these proteins could hydrolyze the naturally-occurring cyanogens. The relationship of these proteins to the enzymes in almonds which hydrolyze amygdalin and prunasin still remains unknown. In summary, although it appears likely that amygdalin hydrolysis in almonds occurs in stepwise fashion with prunasin as intermediate, there is little agreement as to the number and exact nature of β-glycosidases involved in this process.

Employing almond emulsin as the starting-point for β-glycosidase purification may be open to well-founded criticism, since many of the observed isozymes could be merely artefacts produced by the modification of parent β-glycosidases (found in the almond seed) during the preparation of almond

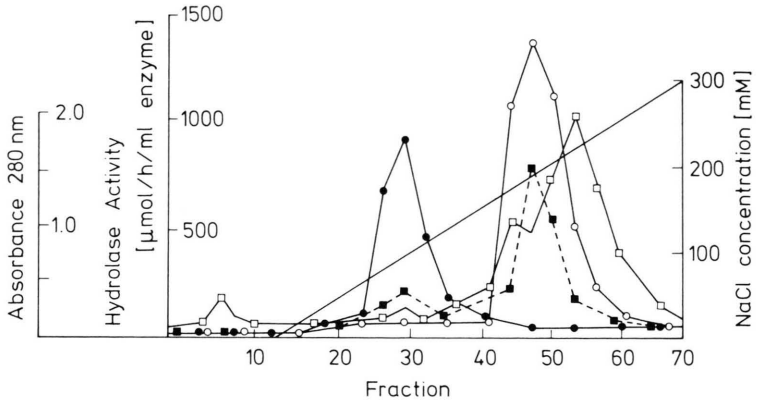

Fig. 2. DEAE-cellulose chromatography of *Prunus serotina* homogenate. Chromatography was undertaken as described under Materials and Methods. Fractions were collected and assayed for AH activity (●—●), PH activity (○—○), and β-glucosidase activity towards β-PNPGlu (■- -■). The protein content was monitored by absorbance at 280 nm (□—□).

emulsin [23, 24]. In view of the unavailability of fresh almonds in Iowa and the uncertainties of utilizing almond emulsin as enzyme source, we have selected seeds of black cherries (harvested from *Prunus serotina* trees grown locally) as our source of β-glycosidases in this study. A crude extract possessing high AH and PH activities was obtained by homogenizing black cherry seeds in sodium acetate buffer as described in the Methods section. Phenolic compounds were removed during homogenization by PVP and by subsequent gel filtration. On applying this preparation to a DEAE-cellulose column in 10 mM histidine-HCl buffer, pH 6.0, both AH and PH activities became bound to the ion-exchanger. Elution of the column with a linear 0–300 mM NaCl gradient effected the complete resolution of these activities (Fig. 2). Fractions eluting between 65–145 mM NaCl showed high activity towards amygdalin but were inactive against prunasin. By contrast, fractions eluting between 160–235 mM NaCl rapidly hydrolyzed prunasin but were virtually inactive against amygdalin. The activity profile for β-PNPGlu hydrolysis closely followed those of AH and PH activities throughout the gradient. The AH and PH fractions were pooled separately and dialyzed overnight to remove sodium chloride prior to the following investigations.

The optimum pH for the hydrolysis of cyanogens was determined using several different buffers. While AH showed maximum activity at pH 5.5, PH was most active at pH 6.5. More than 50% of the maximum rates were realized over the range pH 5.0–7.0. The substrate specificities of the two enzyme preparations were investigated by incubating various potential glycosidic substrates, supplied at 15 mM concentration, with the enzymes at their optimum pH. As shown in Table I, amygdalin hydrolase showed highest activity towards amygdalin. The synthetic substrates β-ONPGlu and β-PNPGlu were hydrolyzed at approximately 50% of the rate observed with amygdalin. Slight activity was observed with β-PNPGal, but the enzyme failed

Table I. Substrate specificity of *Prunus serotina* amygdalin hydrolase and prunasin hydrolase. β-Glycosidase activity was assayed as described in the Methods section using 15 mM concentrations of glycosidic substrates. For comparative purposes, hydrolytic rates shown by amygdalin hydrolase with these substrates are expressed as a percentage of the rate observed with amygdalin as substrate (326 μmol/h/ml enzyme). Hydrolytic rates exhibited by prunasin hydrolase are expressed as a percentage of the rate measured with prunasin (1 540 μmol/h/ml enzyme).

Amygdalin hydrolase		Prunasin hydrolase	
Substrate	% Activity (Amygdalin = 100%)	Substrate	% Activity (Prunasin = 100%)
(*R*)-Amygdalin	100	(*R*)-Prunasin	100
β-ONPGlu	57	β-ONPGlu	79
β-PNPGlu	46	β-PNPGlu	18
β-PNPGal	6	Salicin	3
β-ONPXyl	1	β-PNPGal	1
Maltose	1	Maltose	1
β-PNPXyl	< 1	β-PNPXyl	< 1
(*R*)-Prunasin	0	Cellobiose	< 1
Linamarin	0	Sucrose	< 1
β-Gentiobiose	0	(*R*)-Amygdalin	0
α-PNPGlu	0	Linamarin	0
β-Phenyl-D-glucoside	0	β-Gentiobiose	0
Salicin	0	α-PNPGlu	0
β-Methyl-D-glucoside	0	β-Phenyl-D-glucoside	0
Cellobiose	0	β-Methyl-D-glucoside	0
Sucrose	0	β-PNPXyl	0
Lactose	0	Lactose	0

to hydrolyze a wide range of other substrates including prunasin, linamarin and β-gentiobiose. Prunasin hydrolase was most active with prunasin but also hydrolyzed β-ONPGlu and β-PNPGlu at significant rates. Importantly, this enzyme was inactive towards amygdalin, linamarin, β-gentiobiose and β-phenyl-D-glucoside.

Thus, our findings with black cherry homogenates confirm the conclusion of Haisman and Knight [3] that AH and PH activities in *Prunus* species are catalyzed by two different proteins. It should be noted that, even at this early stage of purification, the black cherry β-glycosidases show surprisingly narrow substrate specificities. Among the physiological glycosidic substrates tested, each enzyme shows a pronounced preference for only one cyanogen and displays little or no activity towards other potential substrates. In view of these strict substrate specificities, we conclude that amygdalin hydrolysis occurs after disruption of black cherries by the sequential mechanism involving stepwise removal of the glucose residues. Non-physiological chromogenic substrates were useful in providing additional information. Both AH and PH hydrolyzed β-PNPGlu significantly, preferring this substrate to the corresponding galactose and xylose derivatives. Their inability to hydrolyze α-glucosides is demonstrated by their lack of activity with α-PNPGlu.

Vicianin hydrolases from V. angustifolia and Davallia species

Vicianin (the β-vicianoside of (*R*)-mandelonitrile) has so far been detected in only two unrelated genera, namely, *Vicia angustifolia* (Leguminosae) [4] and several *Davallia* species (Polypodiaceae) [25]. In contrast to the sequential mode of catabolism observed during amygdalin hydrolysis in black cherries, preliminary experiments by Bertrand *et al.* [4] and Kasai *et al.* [5] indicated that vicianin may be catabolized in *Vicia angustifolia* by the simultaneous mode. Crude enzyme extracts from *V. angustifolia* seeds liberated the disaccharide vicianose during vicianin hydrolysis. In our laboratory, we have investigated the vicianin-hydrolyzing β-glycosidase from *D. trichomanoides* to ascertain whether vicianin might be degraded by a similar mechanism in this taxonomically distinct species.

Homogenization of young *Davallia trichomanoides* fronds with PVP in 50 mM imidazole-HCl buffer, pH 6.0, yielded a cell-free extract which released HCN from the endogenous cyanogen vicianin and also from amygdalin and prunasin. After dialysis, this preparation was applied to a DEAE-cellulose ion-exchange column, and unbound proteins were removed by washing the column. Bound proteins were eluted by a linear 0–200 mM NaCl gradient, and fractions were tested for β-glycosidase activity towards vicianin (as judged by the Feigl-Anger test [8]) and prunasin. As shown in Fig. 3, β-glycosidase activity towards these substrates was eluted under one symmetrical peak by 60–130 mM NaCl. The active fractions were pooled and investigated more closely after removal of NaCl by gel filtration.

The substrate specificity of the pooled β-glycosidase fractions was analyzed by incubating the enzyme preparation with potential substrates (at 10 mM concentration) at the optimum pH (pH 6.0). As shown in Table II, the HPLC technique indicated that vicianin was overall the best substrate

Table II. Substrate specificity of *Davallia trichomanoides* β-glycosidase preparation. β-Glycosidase activity was assayed at pH 6.0 as described in the Methods section, using 10 mM concentration of substrates. Reaction rates were determined in trial 1 by the HPLC technique. In trial 2, the glucose oxidase assay or a standard spectrophotometric test for the hydrolysis of PNP- and ONP-sugars were employed.

Substrate	Trial 1		Trial 2	
%	Reaction rate (μmol/h/ml enz)	% Activity (Vicianin = 100%)	Reaction rate (μmol/h/ml enz)	% Activity (Prunasin = 100%)
(*R*)-Vicianin	168.4	100	0	0
(*R*)-Amygdalin	26.4	16	0.77	3
(*R*)-Prunasin	20.8	12	27.43	100
β-ONPGlu	–	–	6.0	22
β-ONPXyl	–	–	4.41	16
β-PNPGlu	–	–	2.56	9
β-PNPXyl	–	–	1.38	5
β-PNPGal	–	–	0.08	0.3
Lactose			0.05	0.2
α-PNPGlu			< 0.01	0
Salicin	–	–	< 0.01	0
β-Methyl-D-glucoside	–	–	< 0.01	0
Phenyl-β-D-glucoside	–	–	< 0.01	0
Maltose	–	–	< 0.01	0
Cellobiose	–	–	< 0.01	0
β-Gentiobiose	–	–	< 0.01	0
Sucrose	–	–	< 0.01	0
Linamarin	–	–	< 0.01	0

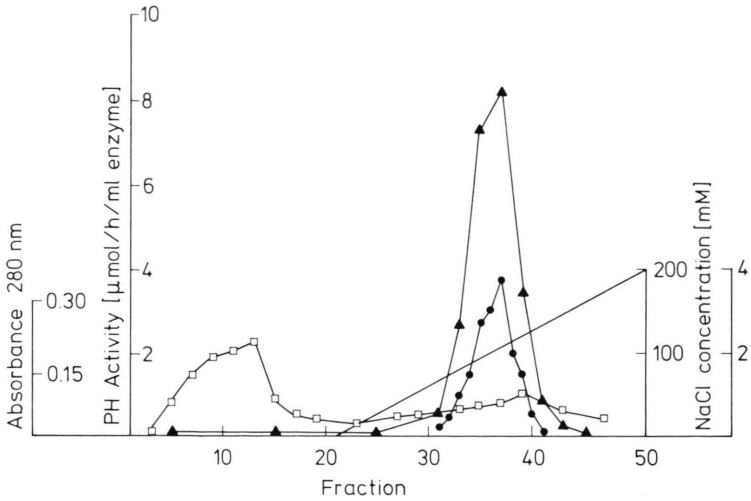

Fig. 3. Ion-exchange chromatography of *Davallia trichomanoides* glycosidase on DEAE-cellulose was undertaken as described in the Methods section. Fractions (3 ml) were assayed for β-glycosidase activity towards vicianin (●—●; determined by a semi-quantitative Feigl-Anger test) and prunasin (▲—▲). The protein content was monitored by absorbance at 280 nm (□—□).

for the *D. trichomanoides* enzyme; amygdalin and prunasin were catabolized at only 16% and 12% of the rate observed with vicianin. In a second trial, hydrolytic rates were determined by the glucose oxidase procedure [9] or by a standard spectrophotometric assay for chromogenic substrates [26]. Among those substrates tested in this trial, prunasin was hydrolyzed most rapidly while much slower rates were observed with the synthetic glycosides β-PNPXyl, β-PNPGlu, β-ONPXyl and β-ONPGlu. Several other glucosidic substrates including salicin, phenyl-β-D-glucoside, β-gentiobiose, α-PNPGlu and the aliphatic cyanogen linamarin were not hydrolyzed by this enzyme preparation. The glucose oxidase test revealed that glucose was released only very slowly when the *D. trichomanoides* β-glycosidase was incubated with amygdalin; this observation did not agree with the rapid release of HCN detected during this reaction by the Feigl-Anger test. Our suspicion that this enzyme hydrolyses amygdalin by a simultaneous mechanism with release of the disaccharide β-gentiobiose was confirmed by thin-layer chromatography as shown in Fig. 4. Neither prunasin nor glucose, which would be the products expected from a sequential hydrolytic mechanism, were detectable. Likewise, hydrolysis of the endogenous substrate vicianin by the *Davallia* enzyme yielded only vicianose as the carbohydrate product; moreover, neither prunasin, glucose nor arabinose were detectable in the terminated reaction mixture. The identities of β-gentiobiose and vicianose as reaction products were confirmed by acidic hydrolysis as described in the Methods section.

In conclusion, we believe that upon disruption of *D. trichomanoides* fronds vicianin would be hydrolyzed by the simultaneous mode, as was described earlier for the *V. angustifolia* β-glycosidase. The release of a disaccharide during hydrolysis of other secondary plant products has also been reported [2].

In summary, the use of endogenous substrates during enzyme purification and characterization has enabled us to demonstrate here the existence of several cyanogen-specific β-glycosidases from plant

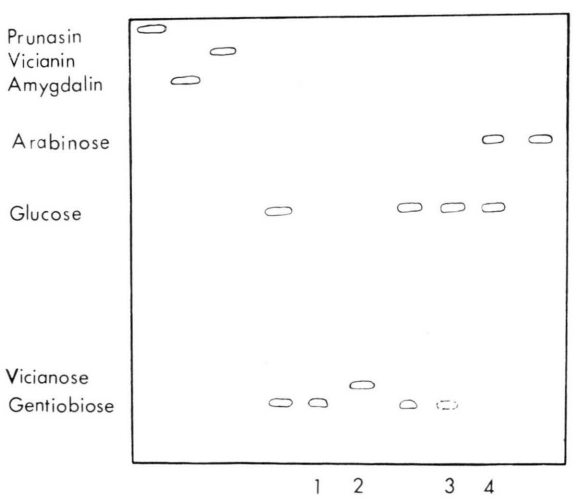

Fig. 4. Schematic diagram showing the formation of disaccharides upon incubation of the *Davallia trichomanoides* β-glycosidase with amygdalin (Lane 1) and vicianin (Lane 2), as detected by the TLC procedure described in the Methods section. The identities of β-gentiobiose and vicianose were confirmed by subsequent acid hydrolysis and TLC analysis (Lanes 3 and 4, respectively).

tissues containing the cyanogenic disaccharides amygdalin and vicianin. In *Prunus serotina*, two distinct proteins, amygdalin hydrolase and prunasin hydrolase, are required for the stepwise hydrolysis of amygdalin by the sequential mechanism. By contrast, the vicianin hydrolase from *Davallia trichomanoides* cleaves vicianin by the simultaneous mode releasing vicianose. The further purification and characterization of these enzymes are in progress in this laboratory.

Acknowledgements

This work has been supported in part by Grant BRSG SO-7 RR7035-17 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, NIH, to J. E. Poulton and a Sigma Xi Grant-In-Aid of Research to P. A. Lizotte. We also wish to thank the University of Iowa for additional funding for HPLC equipment.

[1] E. E. Conn, in: Ann. Rev. Plant Phys. (W. R. Briggs, P. B. Green, and R. L. Jones, eds.), **Vol. 31,** pp. 433–451, Academic Press, New York 1980.
[2] W. Hösel, in: The Biochemistry of Plants: A Comprehensive Treatise (E. E. Conn and P. K. Stumpf, eds.), **Vol. VII,** pp. 725–753, Academic Press, New York 1981.
[3] D. R. Haisman and D. J. Knight, Biochem. J. **103,** 528–534 (1967).
[4] G. Bertrand and L. Rivkid, Bull. Soc. Chim. Biol. **4,** 497–499 (1907).
[5] T. Kasai, M. Kisimoto, and S. Kawamura, Kagawa Daigaku Nogakuba Gakujutso Hokoku **32,** 111–120 (1981).
[6] T. W. M. Fan and E. E. Conn, Plant Physiol. **72,** 128 (1983).
[7] B. Tantisewie, H. W. L. Rujigrok, and R. Hegnauer, Pharm. Weekblad **104,** 1341–1355 (1969).
[8] F. Feigl and V. Anger, Analyst. **91,** 282–284 (1966).
[9] E. Raabo and T. C. Terkildsen, Scand. J. Clin. Lab. Invest. **12,** 402–407 (1960).
[10] B. J. White and J. F. Robyt, Proc. Iowa Acad. Sci., **90,** abstract 83 (1983).
[11] T. Hemscheidt and M. H. Zenk, FEBS Lett. **110,** 187–191 (1980).
[12] S. Veibel, in: The Enzymes (J. B. Sumner and K. Myrback, eds.), **Vol. 1,** pp. 583–620, Academic Press, New York 1950.
[13] W. Hösel and E. E. Conn, TIBS **7,** 219–221 (1982).
[14] A. W. Nahrstedt, W. Hösel, and A. Walther, Phytochemistry **18,** 1137–1141 (1979).
[15] W. Hösel and W. Nahrstedt, Hoppe-Seyler's Z. Physiol. Chem. **356,** 1265–1275 (1975).
[16] S. H. Eklund, M. S. Thesis, University of California, Davis (1981).
[17] G. W. Butler, R. W. Bailey, and L. D. Kennedy, Phytochemistry **4,** 369–381 (1965).
[18] R. Weidenhagen, Ergebn. Enzymforsch. **1,** 197 (1932).
[19] D. R. Haisman, D. J. Knight, and M. J. Ellis, Phytochemistry **6,** 1501–1505 (1967).
[20] P. Lalégerie, Biochimie **56,** 1297–1303 (1974).
[21] F. Helferich and T. Kleinschmidt, Hoppe-Seyler's Z. Physiol. Chem. **340,** 31–36 (1965).
[22] A. K. Grover, D. MacMurchie, and R. J. Cushley, Biochim. Biophys. Acta **482,** 98–108 (1977).
[23] W. D. Loomis, J. D. Lile, R. P. Sandstrom, and A. J. Burbott, Phytochemistry **18,** 1049–1054 (1979).
[24] J. W. Anderson, Phytochemistry **7,** 1973–1988 (1968).
[25] H. Kofod and R. Eyjolfsson, Phytochemistry **8,** 1509–1511 (1969).
[26] M. Gross, G. H. Jacobs, and J. E. Poulton, Anal. Biochem. **119,** 25–30 (1982).