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A β-Glucosidase in Feline Kidney that Hydrolyzes Amygdalin (Laetrile)

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A β-glucosidase has been demonstrated in cat, rat, and rabbit kidney tissue that catalyzes the hydrolytic cleavage of the terminal glucose residue of amygdalin (mandelonitrile-β-gentiobioside). The enzyme was partially purified from feline kidney and its properties were determined. Although the natural substrates for the enzyme are unknown at this time, this β-glucosidase requires an aryl or unsaturated alkyl aglycone moiety for catalytic function. The unusual species and tissue distribution of the enzyme have considerable implications for cancer chemotherapeutic trials with amygdalin (Laetrile).

An intense controversy exists at the present time concerning the use of amygdalin as a cancer chemotherapeutic agent (1–12). The term Laetrile has been used interchangeably with amygdalin (13), although the compound originally referred to as Laterile by Krebs was purportedly the β-glucuronoside of mandelonitrile (1). Here, another controversy exists since it seems unlikely that the procedure employed by Krebs yielded mandelonitrile-β-glucuronide.[1] Amygdalin is the β-gentiobioside derivative of mandelonitrile (Fig. 1). The cancerostatic effect of amygdalin is said to depend primarily upon the release of cyanide following the hydrolytic cleavage of the gentiobiose portion of the molecule. Evidence for the presence of several β-glucosidases in mammalian tissues was obtained several years ago in the course of the isolation of glucocerebroside-β-glucosidase from human placental tissue (14). We report here the partial purification of a β-glucosidase from feline renal tissue that catalyzes the cleavage of the terminal molecule of glucose from amygdalin to yield prunasin. A separate enzyme with different tissue distribution catalyzes the hydrolysis of the monoglucosidic residue of prunasin (mandelonitrile-β-glucoside).

[1] D. M. Rubin, deposition before the United States Food and Drug Administration, Rockville, Md., Sept. 8, 1978.

MATERIALS AND METHODS

Amygdalin (NSC-25122) was supplied by the Drug Synthesis and Chemistry Branch, National Cancer Institute. Prunasin was prepared from amygdalin by modification of the procedure of Fischer (15). Amygdalin (10 g) and 5 g of brewer's yeast "bottom" (Sigma Chemical Col., St. Louis) were dissolved in 100 ml of water. After adding 0.5 ml of toluene, the mixture was shaken at 35°C for 2 days. The reaction was continued for 1 more day with an additional 5 g of yeast. Ethanol (200 ml) was then added and the mixture was filtered. The filtrate was treated with 3 g of neutral activated charcoal for 1 h at 50°C. The charcoal was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in 40 ml of ethyl acetate–methanol–water (12:3:2 v/v). This solution was passed over a 40-g column of E. M. silica gel 60 which had been prepared with ethyl acetate–methanol (4:1 v/v). The column was eluted with 40-ml fractions of the same solvent. The first two fractions, which contained prunasin with only a trace of amygdalin, were evaporated. The residue was recrystallized from 40 ml of ethyl acetate yielding 1.8 g of prunasin mp 150–151°C corr. lit. (16) 147–149°C.

Anal. Calcd for $C_{14}H_{17}NO_6$ (295.28): C, 56.94; H, 5.80; N, 4.74. Found: C, 56.45; H, 5.87; N, 4.24.

The fractions from the chromatography column that contained amygdalin and sugars in addition to prunasin were purified on a similar column using ethyl acetate–methanol (9:1) for elution. The total yield of prunasin was 3.5 g (54%).

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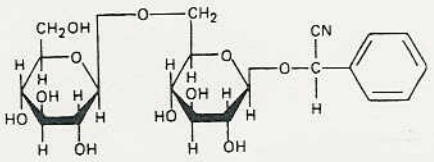

FIG. 1. Structure of amygdalin: (*R*)-α-[(6-*O*-β-D-glucopyranosyl - β - D - glucopyranosyl)oxy]benzene - acetonitrile.

The purity of the glycosides was verified by thin-layer chromatography with ethyl acetate–methanol–water (12:3:2) as the developing solvent. The R_f of prunasin was 0.8, amygdalin 0.6, glucose 0.5, and gentiobiose 0.25. The spots were visualized by charring with sulfuric acid.

Almond β-glucosidase (emulsin) was purchased from Worthington Diagnostics, Freehold, New Jersey.

The quantity of glucose released enzymatically from amygdalin and prunasin was determined with glucose oxidase reagent (Sigma Chemical Co., St. Louis). The reaction was linear between 0 and 110 nmol of glucose. The molecular weight of the β(1 → 6) glucosidase was calculated by gel filtration using a 2.4 × 23-cm column of Sephacryl S-200 (Pharmacia, Piscataway, N. J.). The column was calibrated with ovalbumin (M_r = 45,000), chymotrypsinogen (M_r = 23,200), and cytochrome *c* (M_r = 13,400). Protein was determined by the procedure of Lowry *et al.* (17). Glucocerebrosidase was assayed with ^{14}C-labeled glucocerebroside as described previously (18).

RESULTS

Tissue and Species Distribution of Mammalian β-Glucosidases

All mammalian tissues that have been examined so far have been found to contain glucocerebrosidase activity. Many of these preparations also contain an enzyme that catalyzes the hydrolysis of the β-glucosidic bond of prunasin.[2] However, the enzymatic hydrolysis of the terminal glucose of amygdalin is primarily confined to renal tissue, although it can be detected to a lesser extent in intestinal mucosa preparations.[3] The enzymatic hydrolysis of amydalin could not be demonstrated in rat or human liver, heart, lung, spleen, testis, brain, leukocytes, or cultured skin fibroblasts. Furthermore, the species distribution of this novel glucosidase was sporadic (Table I).

[2] R. O. Brady, A. E. Gal, P. G. Pentchev, and J. A. Barranger, unpublished observations.

[3] R. O. Brady, unpublished observations.

Purification of Feline Kidney β(1 → 6) Glucosidase

The enzyme was found in the high-speed supernatant fraction of aqueous homogenates of cat, rat, and rabbit kidney tissue. Because the specific activity of the enzyme was an order of magnitude greater in the cat kidney preparations than that in the rat or rabbit, feline tissue was used for the isolation of the enzyme. The enzyme was concentrated by 45 to 60% saturation of the supernatant solution with ammonium sulfate. The precipitated protein was taken up in water and the solution was applied to a 2.4 × 23-cm column of Sephacryl S-200 that had been equilibrated with 25 mM-sodium phosphate buffer solution (pH 6.5) containing 0.1 M NaCl. The column was developed with the same buffered salt solution and 2-ml fractions were collected at a flow rate of 1 ml/min. The enzyme was enriched in fractions 33 to 38. These fractions were pooled and treated in batchwise fashion with a suspension of hydroxylapatite (0.42 mg of gel ml^{-1}) by adding 1 ml of the suspended gel to 1.5 ml of enzyme solution. The suspension was centrifuged and the supernatant

TABLE I

SPECIES DISTRIBUTION OF MAMMALIAN KIDNEY β-GLUCOSIDASES[a]

Species	Substrate hydrolyzed (nmol/mg of protein/h)		
	Amygdalin	Prunasin	Glucocerebroside
Cat	99.	118.	12.
Rat	8.3	4.1	62.
Rabbit	6.3	25.	27.
Human	0.0	0.25	43.
Mouse	0.0	0.0	20.
Guinea pig	0.0	0.0	28.
Sheep	0.0	0.0	nd[b]
Bovine	0.0	nd	nd

[a] Minced kidney tissue was homogenized in an all-glass homogenizer in 10 vol (w/v) of cold distilled water. Aliquots were incubated with amygdalin (27.3 mM) or prunasin (42.4 mM) in 0.05 M sodium phosphate buffer (pH 7.5) for 18 h at 37°C. Glucocerebrosidase activity was determined as described previously (18).

[b] Not determined in this series.

TABLE II

PURIFICATION OF CAT KIDNEY $\beta(1 \rightarrow 6)$ GLUCOSIDASE

Step	Volume (ml)	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
(1) Aqueous homogenate	18.	37,300	284.	131.		
(2) 100,000*g* Supernatant	14.	30,100	90.	334.	2.6	81.
(3) Ammonium sulfate, 0.45–0.60 saturation	4.4	15,050	26.	579.	4.4	40.
(4) Sephacryl S-200	12.	10,200	4.7	2,170.	17.	27.
(5) Hydroxylapatite, 0.1 M phosphate eluate	1.8	3,690	0.46	8,020.	61.	9.9

[a] One unit of enzymatic activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 nmol of amygdalin/h using the assay conditions described in Table I.

solution was decanted. The precipitate was washed with 1.8-ml aliquots of 25, 50, and 100 mM sodium phosphate buffer solution pH 6.5. The majority of the enzymatic activity was recovered in the 100 mM phosphate eluate. A 61-fold enrichment of the enzyme was obtained in a yield of 9.9% (Table II).

Properties of Feline Kidney $\beta(1 \rightarrow 6)$ Glucosidase

The optimal pH of the enzyme is 7.5 (Fig. 2). The reaction was linear with time over a period of 22.5 h at 37°C (Fig. 3). The reaction was proportional to the amount of protein in the incubation mixtures over a range of 1.2 to 7.2 μg of protein (Fig. 4). The K_m of amygdalin is 3.8×10^{-3} M (Fig. 5). The M_r estimated by gel filtration was 32,000.

The enzyme was not adsorbed by concanavalin A–Sepharose. Catalytic activity was suppressed in the presence of Tris buffer. The addition of the divalent cations Mg^{2+} and Ca^{2+} had no effect on the hydrolysis of amygdalin. The enzymatic activity was destroyed by heating at 100°C for 10 min and by incubating the enzyme in the presence of trypsin or *S. griseus* protease (Sigma Chemical Co.). Most (93%) of the amygdalin hydrolyzing activity in cat kidney tissue was inactivated when the tissue was frozen prior to homogenization.

An unexpected observation made in the course of this investigation was the complete inactivity of the $\beta(1 \rightarrow 6)$ glucosidase with gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose). In fact, we were unable to demonstrate the hydrolysis of gentiobiose in any of the mammalian kidney preparations we

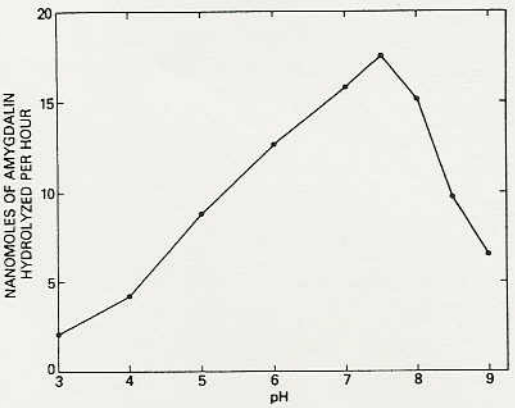

FIG. 2. Effect of pH on the hydrolysis of amygdalin by kidney $\beta(1 \rightarrow 6)$ glucosidase. Citrate–phosphate buffer was used at pH 3, sodium acetate buffer at pH 4 and 5, sodium phosphate buffer at pH 6 to 8, and sodium barbital buffer at pH 8.5 and 9. The final concentration of the various buffers was 50 mM. Enzyme from step 2, Table II (0.64 mg of protein), was incubated with 27 mM amygdalin for 18 h at 37°C in a final volume of 0.2 ml. The reaction was stopped by the addition of 0.06 ml each of 0.3 N $Ba(OH)_2$ and 0.3 N $ZnSO_4$. The suspensions were centrifuged and the glucose liberated was determined as described under Materials and Methods. Because up to 4% of the amygdalin was hydrolyzed in the experiments shown in this and the subsequent figures, a fraction of the glucose produced may have arisen from the activity of prunasinase in the preparations.

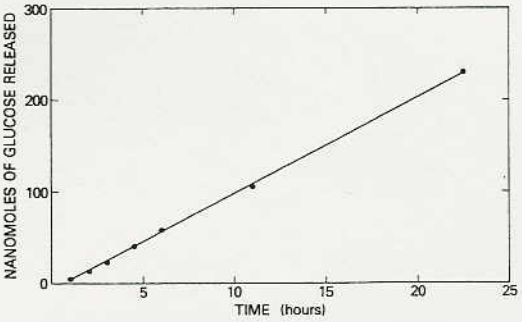

FIG. 3. Hydrolysis of amygdalin as a function of time. Aliquots of enzyme from step 4, Table II (6.5 μg of protein), were incubated with 27 mM amygdalin for varying periods of time at 37°C in 0.05 M sodium phosphate buffer (pH 7.5). The reaction was stopped and glucose was determined as described in the legend to Fig. 2.

tested (sheep and bovine kidney were not examined). The obscure nature of the natural substrate(s) of the feline, rat, and rabbit kidney β(1 → 6) glucosidase is further attested by our finding that gentianose [*O*-β-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside] was not hydrolyzed by unfractionated kidney homogenates that exhibited abundant activity with both amygdalin and prunasin. On the other hand, crocin [8,8′-diapo-ψ,ψ-carotenedioc acid bis(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)ester] was readily hydrolyzed by the enzyme although the extent of the reaction was not investigated.

Evidence that the Enzymes that Catalyze the Hydrolysis of Amygdalin and Prunasin Are Distinct Proteins

The cold instability of the β-glucosidase that catalyzes the hydrolysis of the terminal β-glucosidic bond of amygdalin differs significantly from that exhibited by the enzyme that catalyzes the cleavage of glucose from prunasin since virtually all of the catalytic activity of the latter enzyme is retained on freezing the source tissue. This difference in cold lability was also observed when whole homogenates of both cat and rat kidney were frozen. Again, the loss of amygdalin hydrolyzing enzyme activity was greater than that of the prunasin glucosidase. Further evidence of the discreteness of these enzymes is seen in the change in the relative rate of hydrolysis of these glucosides upon enrichment of the β(1 → 6) glucosidase. The ratio of amygdalin cleaved to prunasin in a whole aqueous homogenate was 0.7:1. This ratio increased to 2.1:1 in the enzyme eluted from hydroxyl apatite. Furthermore, the ratio in the unfractionated homogenate may be an overestimate of the true value since it seems likely that some of the prunasin produced by the β(1 → 6) glucosidase when amygdalin was the substrate was further cleaved by the prunasin hydrolyzing enzyme in this preparation. Because of the presence of residual prunasin hydrolytic activity in our most highly enriched amygdalin cleaving fraction, we have not characterized the products of the β(1 → 6) glucosidase.

DISCUSSION

We have demonstrated the presence of a β(1 → 6) glucosidase in cat, rat, and rabbit kidney tissue. Because the enzyme was most active in the feline kidney preparations, partial purification of the β-glucosidase was carried out using this tissue. Several unusual characteristics of the β-glucosidase are noteworthy. The enzyme is primarily found in the cytosol and has a neutral pH optimum. These properties contrast with the lysosomal localization of many

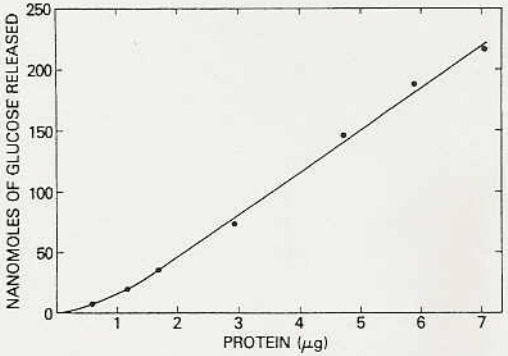

FIG. 4. Proportionality between glucose released and enzyme protein. Aliquots of enzyme from step 4, Table II, were incubated with 27 mM amygdalin at pH 7.5 for 18 h at 37°C. The reaction was stopped and free glucose determined as in Fig. 2.

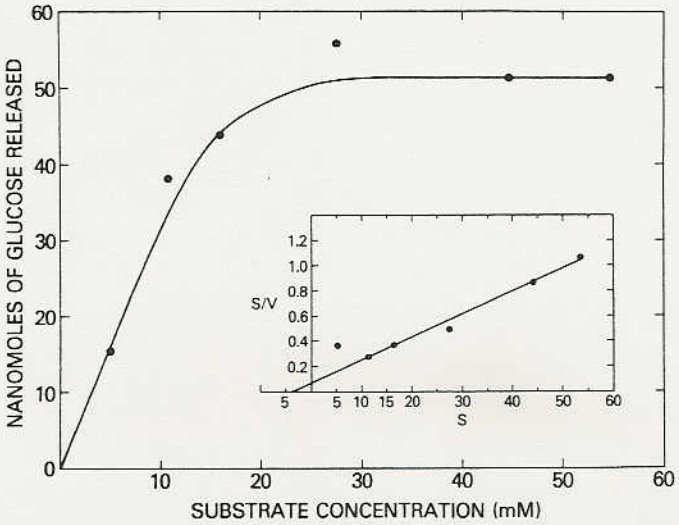

FIG. 5. The hydrolysis of amygdalin as a function of substrate concentration. Aliquots of enzyme from step 4, Table II (2.2 μg of protein), were incubated with varying quantities of amygdalin for 18 h at 37°C. The inset is a plot of the ratio of substrate concentration to velocity (S/V) versus substrate (S).

mammalian glycosidases and their acidic pH optima (19, 20). The natural substrate(s) of the enzyme is unresolved. This problem appears complicated by the fact that the hydrolysis of the disaccharide gentiobiose, which is an integral part of the structure of amygdalin, could not be demonstrated in any mammalian tissue examined in the course of this investigation. A requirement for an aryl or unsaturated alkyl aglycone moiety is also suggested from the inability of these preparations to catalyze the hydrolysis of gentianose. Although amygdalin occurs naturally in bitter almonds, peaches, and apricots, it seems difficult to reconcile this distribution with high activity in feline kidney. This situation is in contrast with almond β-glucosidase(s). Both molecules of glucose are cleaved from amygdalin by the plant enzyme and prunasin and gentiobiose are good substrates for almond glucosidase(s) (data not shown).

An approach to the therapy of malignant disease that is based on the liberation of cyanide *in situ* must take into consideration the presence or absence of enzyme(s) that unblocks the bound cyano moiety. This reservation may not be a major concern when the β-glucuronide derivative of mandelonitrile is employed since most tissues exhibit high β-glucuronidase activity (21). On the other hand, the use of amygdalin presents a serious problem in this regard since we have found that most mammalian tissues do not hydrolyze the terminal $\beta(1 \rightarrow 6)$ glucosidic bond of this compound. The most striking exception is kidney, and even here, the species specificity of the $\beta(1 \rightarrow 6)$ glucosidase is puzzling. For example, the enzyme can be demonstrated in rat kidney, but not in mouse kidney. In contrast, the hydrolysis of prunasin occurs readily in many mammalian tissues in addition to kidney. Although prunasin is a $\beta(1 \rightarrow 1)$ glucoside, as is glucocerebroside, it seems likely from the data in Table I that separate enzymes catalyze the hydrolysis of these substances. Thus, our evidence indicates that mammalian tissues contain at least three distinct β-glucosidases. Attempts to devise chemotherapeutic agents that depend on the release of cyanide following the cleavage of glucosidic bonds should not only take careful account of the restricted species and tissue distribution of the required enzymes but also include a determination of their presence in the neoplastic tissue.

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