Plant Physiol. (1992) 100, 282–290
0032-0889/92/100/0282/09/$01.00/0

Received for publication January 15, 1992
Accepted May 4, 1992

Prunus serotina Amygdalin Hydrolase and Prunasin Hydrolase[1]

Purification, N-Terminal Sequencing, and Antibody Production

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ABSTRACT

In black cherry (*Prunus serotina* Ehrh.) seed homogenates, amygdalin hydrolase (AH) participates with prunasin hydrolase (PH) and mandelonitrile lyase in the sequential degradation of (*R*)-amygdalin to HCN, benzaldehyde, and glucose. Four isozymes of AH (designated AH I, I′, II, II′) were purified from mature cherry seeds by concanavalin A-Sepharose 4B chromatography, ion-exchange chromatography, and chromatofocusing. All isozymes were monomeric glycoproteins with native molecular masses of 52 kD. They showed similar kinetic properties (pH optima, K_m, V_{max}) but differed in their isoelectric points and N-terminal amino acid sequences. Analytical isoelectric focusing revealed the presence of subisozymes of each isozyme. The relative abundance of these isozymes and/or subisozymes varied from seed to seed. Three isozymes of PH (designated PH I, IIa, and IIb) were purified to apparent homogeneity by affinity, ion-exchange, and hydroxyapatite chromatography and by nondenaturing polyacrylamide gel electrophoresis. PH I and PH IIb are 68-kD monomeric glycoproteins, whereas PH IIa is dimeric (140 kD). The N-terminal sequences of all PH and AH isozymes showed considerable similarity. Polyclonal antisera raised in rabbits against deglycosylated AH I or a mixture of the three deglycosylated PH isozymes were not monospecific as judged by immunoblotting analysis, but also cross-reacted with the opposing glucosidase. Monospecific antisera deemed suitable for immunocytochemistry and screening of expression libraries were obtained by affinity chromatography. Each antiserum recognized all known isozymes of the specific glucosidase used as antigen.

Several thousand plant species, including many economically important food plants, accumulate cyanogenic glycosides and cyanolipids (22). Upon tissue disruption, these natural products are catabolized to the respiratory poison HCN and simple carbonyl compounds. A role for these catabolites in plant protection against herbivory has received considerable experimental support (11, 18). The most well-known cyanogenic glycoside is (*R*)-amygdalin [the β-gentiobioside of (*R*)-mandelonitrile], which occurs in high concentrations in seeds of rosaceous stone fruits (e.g. bitter almonds, apricots, peaches). Our laboratory has shown that, when black cherry (*Prunus serotina*) seeds are crushed, amygdalin is rapidly degraded to benzaldehyde, glucose, and HCN by the sequential action of the enzymes AH[2], PH, and MDL (12, 29). One of the complexities of the *Prunus* cyanogenic system yet to be satisfactorily unraveled is the microheterogeneity shown by these catabolic enzymes. Earlier studies (13, 14, 29) established that AH and PH occur in cherry homogenates as two and three isozymes, respectively, whose existence was not attributable to partial proteolysis during isolation. The nature and physiological significance of this multiplicity might best be approached by N-terminal sequencing followed by construction and screening of a black cherry cDNA expression library. As with other cyanogenic species, large-scale cyanogenic glucoside catabolism in *P. serotina* occurs only after tissue disruption, implicating some crucial compartmentation of amygdalin and its catabolic enzymes in undamaged seeds. However, such spatial regulation of cyanogenesis remains poorly understood (27, 28).

In this paper, we describe how previous purification procedures have been improved to achieve the isolation of four AH and three PH isozymes for use in N-terminal sequencing. Monospecific polyclonal antibodies have been generated against AH and PH for future use in immunocytochemical localization studies and screening of expression libraries. Additionally, to gain further insight into the multiplicity shown by AH, we have devised a simple protocol involving affinity chromatography and IEF to screen rapidly AH isozyme patterns of individual seeds.

MATERIALS AND METHODS

Plant Materials

Mature black cherry (*Prunus serotina* Ehrh.) fruits were collected from trees growing at different sites in Iowa City, Iowa. Pits (endocarp enclosing seed) were freed from exocarp and fleshy mesocarp tissues, surface sterilized with 0.52% (w/v) sodium hypochlorite for 20 min, and rinsed thoroughly with distilled water. Finally, they were blotted dry on paper towels and stored in sealed plastic containers at 4°C until used.

[1] Supported by National Science Foundation grant DCB 89-17176

[2] Abbreviations: AH, amygdalin hydrolase; PH, prunasin hydrolase; MDL, mandelonitrile lyase; 4-MUG, 4-methylumbelliferyl-β-D-glucoside; TBS, Tris-buffered saline; TFMS, trifluoromethane sulfonic acid; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; IgG, immunoglobulin G; pI, isoelectric point.

Biochemicals and Chromatographic Materials

The following chemicals were obtained from Sigma: (*R*)-amygdalin, (*R*)-prunasin, *p*-nitrophenyl-β-D-glucoside, 4-MUG, BSA, glucose oxidase reagents, polyvinylpolypyrrolidone, Con A-Sepharose 4B, periodic acid-Schiff reagent, methyl α-D-glucoside, TFMS, Freund's adjuvants, and Reactive Red 120-agarose. Protein determination reagents were obtained from Bio-Rad (Richmond, CA). Chromatographic materials were purchased from the following sources: DEAE-cellulose (DE-52) and CM-cellulose (CM-52), Whatman Chemical Separation Ltd. (Kent, UK); HA Ultragel (hydroxyapatite), IBF Biotechnics (Columbia, MO); Polybuffer Exchanger 94, Polybuffer 74, Phastgel IEF 3/9 gels, broad range pI markers, Phastgel silver staining kit, FPLC Superose 12 (HR 10/30) column, and CNBr-activated Sepharose 4B, Pharmacia Fine Chemicals (Piscataway, NJ).

Purification of AH

All procedures were undertaken at 4°C. Three hundred black cherry seeds (approximately 30 g), collected from several trees, were homogenized with a pestle and mortar with 4 g of polyvinylpolypyrrolidone, 8 g of quartz sand, and 60 mL of buffer A (0.1 M histidine-HCl buffer, pH 6.0). The homogenate was filtered through four layers of cheesecloth and centrifuged for 25 min at 17,600*g*. The supernatant was decanted with minimal disturbance of the lipid layer that formed during centrifugation and was dialyzed overnight against 4 L of buffer B (10 mM histidine-HCl, pH 6.0, containing 0.17 M NaCl). The dialyzed enzyme solution was applied to a Con A-Sepharose 4B column (1 × 16 cm) preequilibrated with buffer B. After extensive washing with buffer B, bound proteins were eluted with the same buffer containing 0.2 M methyl α-D-glucoside. Fractions containing AH activity were pooled and dialyzed overnight against 4 L of buffer C (20 mM sodium acetate-HCl buffer, pH 5.0). The resulting preparation was centrifuged for 5 min at 17,600*g* before passage through a DEAE-cellulose (1 × 16 cm) column, previously equilibrated with buffer C. The eluate, now lacking PH and MDL, was applied to a CM-cellulose column (1 × 5 cm) preequilibrated with buffer C. After washing the column extensively with the same buffer, bound proteins including AH were eluted with buffer C containing 0.15 M NaCl. Fractions displaying AH activity were pooled and dialyzed overnight against buffer D (25 mM imidazole-HCl, pH 7.4). The dialyzed preparation was applied to a Polybuffer Exchanger 94 chromatofocusing column (1 × 40 cm) preequilibrated with buffer D. Proteins retained on the column were eluted with buffer E (Polybuffer 74 previously diluted 1:8 with distilled water and adjusted to pH 5.0 with HCl). Fractions containing different AH isozymes were pooled individually, dialyzed overnight against 4 L of buffer F (10 mM histidine-HCl, pH 6.0, containing 0.04% [w/v] NaN_3), and stored at 4°C.

Purification of PH

All operations were performed at 4°C. PH was isolated from black cherry seeds and subjected to Con A-Sepharose chromatography essentially as described above for AH. Proteins released from that affinity matrix by 0.2 M methyl α-D-glucoside were dialyzed overnight against 4 L of buffer C and applied to a DEAE-cellulose column (1.6 × 12 cm) preequilibrated with buffer C. After washing with 10 column volumes of buffer C, bound proteins were eluted with 75 mL of buffer C containing 0.2 M NaCl. The resulting protein peak, which contained PH, was dialyzed against 4 L of buffer C and applied to a Reactive Red-120 agarose column (1.6 × 22 cm) preequilibrated with buffer C. After sample application, the column was allowed to stand at least 30 min before washing with 10 column volumes of buffer C. Fractions exhibiting PH activity that did not bind to the affinity matrix were pooled and dialyzed overnight against 4 L of buffer G (3 mM sodium phosphate, pH 6.8). PH that bound to Reactive Red-120 was eluted with a linear gradient (400 mL) of 0 to 0.2 M NaCl in buffer C. Active fractions were pooled and dialyzed against buffer G. Each of the two dialyzed pools resulting from reactive red chromatography was concentrated to 5 mL (Amicon Ultrafiltration Cell 52) and applied to individual HA Ultragel columns (1 × 16 cm) preequilibrated with buffer G. Columns were washed with 400 mL of buffer G and fractions (5 mL) were collected. Elution continued with linear gradients (300 mL) of 3 to 250 mM sodium phosphate, pH 6.8, and fractions (5 mL) were collected.

In keeping with previous designations (14), PH activity failing to bind to hydroxyapatite is referred to as PH I, and PH activity retained by this column (prior to gradient application) is designated PH II. The PH I and II pools were individually concentrated to 1 mL by ultrafiltration and subjected to nondenaturing PAGE (2) at pH 8.0 on separate 8% gels (1.5 mm thick). PH bands were detected by activity staining with 4-MUG, excised, and individually electroeluted using an ISCO 1750 sample concentrator (electrode buffer, 40 mM Tris-acetate, pH 8.6; sample buffer, a 1:4 dilution thereof). Eluates were dialyzed overnight against 4 L of double-distilled H_2O and concentrated to 1 mL by ultrafiltration. Concentrates were lyophilized and stored at −20°C until used.

Gel Electrophoresis

Analytical SDS-PAGE was performed by the method of Laemmli (15). Gels (10%) were run at 10 mA (constant current) per gel at 15°C. Two-dimensional SDS-PAGE was performed with a Protean II xi system (Bio-Rad) according to O'Farrell (20) using Ampholine 5/7 (LKB, Uppsala, Sweden) and Pharmalyte 2D 3/10 (Pharmacia, Uppsala, Sweden) as ampholytes in a ratio of 9:1. Proteins were detected by brilliant blue G-colloidal staining (19). Periodic acid-Schiff staining of glycoproteins was performed according to Segrest and Jackson (23).

IEF was performed on PhastGel IEF 3/9 gels at 15°C using the Pharmacia Phast system. Total proteins were visualized using a silver staining kit according to manufacturer's instruction. For activity staining, the fluorogenic substrate 4-MUG was used to locate AH on native IEF gels. Gels were examined under UV light after 5 min incubation in 0.2 M sodium acetate buffer, pH 4.75, containing 5 mM 4-MUG. This procedure was also used to locate the three PH isozymes after nondenaturing PAGE (2).

Protein Determinations and Other Analytical Methods

Protein was determined by the Bradford procedure (1) using a Bio-Rad protein assay kit with BSA serving as standard. AH and PH activities were assayed as previously described (13, 14). Amino-terminal sequences were determined at the University of Iowa Protein Structure Facility by Edman degradation on an Applied Biosystems 475A protein sequenator. The native molecular mass of purified AH was determined by FPLC on a Superose 12 (HR 10/30) gel filtration column using 0.1 M sodium phosphate buffer, pH 7.4, as eluent at a flow rate of 0.8 mL/min. Calibration markers were thyroglobulin (670 kD), gamma globulin (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and vitamin B-12 (1.35 kD).

Production of Antisera against Deglycosylated AH and PH

Polyclonal antisera were raised in male New Zealand white rabbits against TFMS-deglycosylated (26) proteins. Two weeks after collection of preimmune sera, rabbits were immunized at multiple subcutaneous sites along the backbone with deglycosylated AH I (200 μg) or a mixture containing equivalent amounts of deglycosylated PH I, IIa, and IIb (300 μg of total protein) that had been emulsified with equal volumes of Freund's complete adjuvant. Booster injections (100 μg of deglycosylated AH I after 21 d and 56 d; 300 μg of deglycosylated PH isozyme mixture after 21 d) were administered in Freund's incomplete adjuvant. Rabbits were bled from marginal ear veins 11 to 14 d after the final booster injection. IgG-enriched antisera were obtained from whole blood by centrifuging for 10 min at 1500*g* and heating the supernatant serum at 55°C for 15 min to inactivate complement, followed by ammonium sulfate precipitation (4). These antisera were dialyzed overnight against 4 L of 20 mM potassium phosphate, pH 7.4, containing 0.9% (w/v) NaCl and 0.02% (w/v) sodium azide and stored as small aliquots (0.5 mL) at −20°C until used. IgG-enriched antisera were prepared from preimmune sera by identical procedures.

Affinity Purification of Anti-AH Antisera

Antibody purification was achieved by passing anti-AH antisera (initial volume, 1 mL) twice through a Con A-Sepharose 4B column (1 × 2 cm) previously loaded with PH and MDL (approximately 10 mg of total protein that had been freed of AH by DEAE-cellulose chromatography). The preequilibration and elution buffer was 10 mM histidine-HCl buffer, pH 6.0, containing 1% (w/v) NaCl. To obtain control sera, preimmune serum was similarly treated.

Affinity Purification of Anti-PH Antisera

Black cherry seed proteins (35 mg) that failed to bind to Con A-Sepharose were conjugated to 7 mL packed volume of CNBr-activated Sepharose 4B according to the manufacturer's instructions. A column (1 × 5 cm) was constructed and equilibrated with PBS (20 mM potassium phosphate, pH 7.4, containing 0.2 M NaCl). The IgG-enriched antiserum (2 mL), diluted 1:10 with PBS, was passed three times through this column. For further antibody purification, proteins (1 mg total) separated from PH by DEAE-cellulose (nonbinding proteins) and Reactive Red 120-agarose (eluted by 0.2–1 M NaCl gradient in buffer C) chromatography were applied to a nitrocellulose membrane (3 × 6 cm). After being air dried, the membrane was incubated overnight with blocking solution (20 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl, 0.05% [w/v] Tween 20, and 5% nonfat dry milk) to block nonspecific binding sites. The membrane was washed three times with double-distilled water and incubated with the partially purified antiserum for 4 h at 25°C. After discarding the membrane, the purified antiserum was stored in small aliquots (1 mL) at −20°C. To obtain control sera, preimmune sera were similarly treated.

Immunochemical Analyses

Antisera titers were determined by dot immunobinding assays (7). Antisera specificities were analyzed by immunoblotting techniques. Crude cherry homogenates as well as homogeneous AH and PH were subjected to one-dimensional (15) and two-dimensional SDS-PAGE (20). Proteins were electroblotted onto nitrocellulose membranes with a Bio-Rad Trans-Blot apparatus and probed using specific polyclonal antibodies as previously described (27). In control blots, anti-AH and anti-PH antisera were replaced by preimmune serum.

Isozyme Patterns in Individual Seeds

All procedures were undertaken at 4°C. Individual seeds were homogenized with a pestle and mortar in 0.5 mL of buffer B. After centrifugation in a Beckman Microfuge for 5 min, the supernatant liquid was incubated for 1 h with 60 μL of packed volume of Con A-Sepharose 4B (preequilibrated with buffer B) under gentle agitation. The affinity matrix was recovered by centrifugation and washed four times with the above buffer before releasing the bound glycoproteins by incubation with 0.5 mL of 0.2 M methyl α-D-glucoside in buffer B. After separating the matrix by centrifugation, proteins in the supernatant were subjected to IEF on Phastgel IEF 3/9 gels.

RESULTS AND DISCUSSION

Purification and Properties of Four AH Isozymes

Upon disruption of mature black cherry seeds, amygdalin hydrolase initiates the catabolism of (*R*)-amygdalin to HCN. Four isozymes of AH have now been extensively purified from this source by Con A-Sepharose 4B chromatography, ion-exchange chromatography, and chromatofocusing. These isozymes were designated AH I′, AH I, AH II, and AH II′ in order of their elution during chromatofocusing (Fig. 1). Each isozyme was purified approximately 200-fold (Table I). Upon FPLC gel filtration, individual isozymes, as well as a mixture of isozymes, showed a single, symmetrical protein peak corresponding to a native molecular mass of approximately 52 kD. On 10% SDS-PAGE gels, the isozymes appeared as a single polypeptide band of molecular mass 60 kD, suggesting that they are monomeric. Upon analytical IEF, AH I′, AH I, AH II, and AH II′ displayed pI values of approximately 6.4,

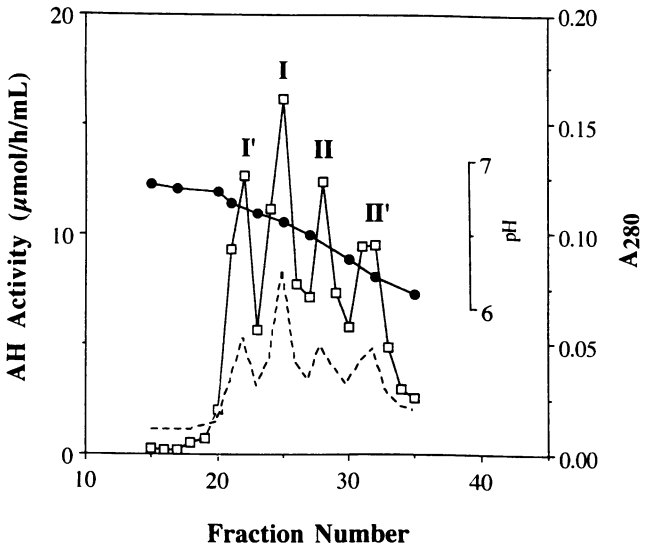

Figure 1. Chromatofocusing of *P. serotina* AH isozymes. Chromatofocusing was performed as described in "Materials and Methods" on a Polybuffer Exchanger 94 column (1 × 40 cm) using Polybuffer 74 as eluent. Protein concentration (- - -) was continuously monitored at A_{280} using an ISCO V^4 absorbance detector. Fractions (5 mL) were assayed for AH activity (□ - □) and pH (● - ●).

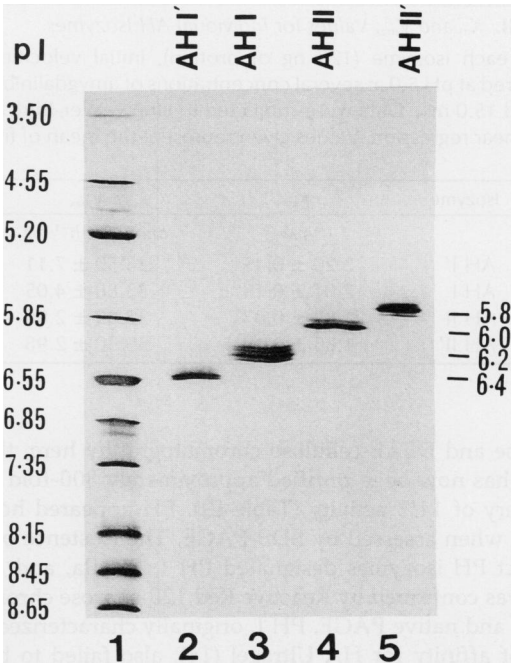

Figure 2. IEF analysis of *P. serotina* AH isozymes. Individual isozymes, previously resolved by chromatofocusing, were subjected to IEF on a Phastgel IEF 3/9 gel and detected by silver staining. Lane 1, broad-range pI markers (Pharmacia); lanes 2 to 5, individual AH isozymes as indicated.

6.2, 6.0, and 5.8, respectively (Fig. 2). However, each isozyme preparation showed more than one band. Such subisozymes, whose β-glycosidic activity was established by activity staining using the fluorogenic substrate 4-MUG, were not resolved by chromatofocusing, probably because of extreme closeness in their pI values (i.e. differing by less than 0.05 pH unit). The glycoprotein nature of AH isozymes was demonstrated by their positive periodic acid-Schiff staining (data not shown) and by their ability to bind to Con A-Sepharose 4B with subsequent elution by methyl α-D-glucoside.

The effect of pH on catalysis by individual AH isozymes was determined at different pH values in citrate-potassium phosphate buffer. All isozymes displayed maximum activity in the range pH 4.5 to 5.5 (data not shown). This value is similar to the pH optima of other plant β-glycosidases involved in cyanogenesis (10, 14, 21, 24). At pH 5.0, the AH isozymes displayed insignificant differences in their K_m and V_{max} values toward their endogenous substrate amygdalin (Table II). Among many naturally occurring glycosides tested, AH I and AH II hydrolyzed amygdalin most rapidly (13).

Purification of Three PH Isozymes

PH, which catalyzes the further hydrolysis of (*R*)-prunasin to mandelonitrile and D-glucose, was extensively purified by Kuroki and Poulton (14) using Con A-Sepharose 4B and HA Ultragel chromatography. By including Reactive Red 120-

Table I. *Summary of the Purification of AH Isozymes from P. serotina Seeds*

The four AH isozymes were purified from mature black cherry seeds as described in "Materials and Methods." The following values are representative of five separate purifications.

Purification Step	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	mg	*units*[a]	*units mg*$^{-1}$	%	*-fold*
Crude homogenate	1936.6	267.4	0.14	100.0	
Con A-Sepharose 4B	9.9	73.5	7.43	27.5	53.1
CM-cellulose	1.6	36.5	22.83	13.7	163.1
Chromatofocusing					
AH I'	0.3	8.7	29.00	3.3	207.1
AH I	0.5	17.6	35.20	6.6	251.4
AH II	0.3	8.7	29.00	3.3	207.1
AH II'	0.3	8.1	27.00	3.0	192.9

[a] One unit of enzyme activity yields 1 mmol glucose h^{-1} under standard assay conditions.

Table II. *K_m and V_{max} Values for Individual AH Isozymes*

For each isozyme (120 ng of protein), initial velocities were measured at pH 5.0 at several concentrations of amygdalin between 0.9 and 15.0 mM. Data were subjected to Lineweaver-Burk analysis with linear regression. Values given represent the mean of five trials ± SD.

Isozyme	K_m	V_{max}
	mM	mmol Glc h^{-1} mg^{-1}
AH I′	2.20 ± 0.45	33.50 ± 7.11
AH I	2.01 ± 0.19	33.80 ± 4.05
AH II	1.88 ± 0.07	37.89 ± 2.96
AH II′	1.65 ± 0.19	30.30 ± 2.98

agarose and DEAE-cellulose chromatography here, this enzyme has now been purified approximately 800-fold with a recovery of 11% activity (Table III). PH appeared homogeneous when assessed by SDS-PAGE. The existence of three distinct PH isozymes designated PH I, PH IIa, and PH IIb (14) was confirmed by Reactive Red 120-agarose chromatography and native PAGE. PH I, originally characterized by its lack of affinity for HA Ultragel (14), also failed to bind to Reactive Red 120-agarose. In contrast, PH II was retained by both HA Ultragel and reactive red 120-agarose and was further resolved into dimeric (PH IIa) and monomeric (PH IIb) isozymes by nondenaturing PAGE (Fig. 3). This procedure allowed superior resolution of these isozymes than was previously achieved by gel filtration (14).

Thus, *P. serotina* AH and PH display many physical properties common to the majority of cyanogenic β-glycosidases such as existing as multiple forms and having glycoprotein character, an acidic pI, and a subunit molecular mass of 50 to 65 kD (21).

Isolation and Characterization of Anti-AH and Anti-PH Antisera

Anticipating that polyclonal antisera raised in rabbits against native AH and PH might cross-react with other glycoproteins having similar glycans (16), we deglycosylated these glucosidases by TFMS before immunization. Upon deglycosylation, the apparent molecular mass of AH I decreased by approximately 8% as seen by SDS-PAGE. Antisera obtained by immunizing rabbits with deglycosylated AH I not only recognized all AH isozymes but also reacted strongly toward PH, suggesting that these glycosidases might share common epitopes (Fig. 4). Also, some cross-reaction was seen toward MDL. Because its specificity was insufficient for immunocytochemical localization studies, the antiserum was further purified by passage through a Con A-Sepharose 4B column that had been preloaded with PH and MDL. Figure 4A clearly demonstrates the efficacy of this procedure. Although its titer decreased 5-fold, the purified anti-AH antiserum was monospecific for AH as shown by western immunoblotting after one-dimensional (Fig. 4A) and two-dimensional SDS-PAGE (Fig. 5). Furthermore, slot-blot immunoassay (Fig. 6) as well as two-dimensional SDS-PAGE immunoanalysis showed that the purified antiserum recognized all four AH isozymes. As little as 50 ng of AH protein were detectable in slot-blot assays.

SDS-PAGE analysis indicated that deglycosylated PH formed high mol wt aggregates (data not shown). Antisera obtained by immunizing rabbits with a mixture of all three

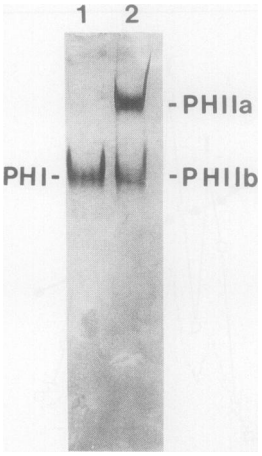

Figure 3. Nondenaturing PAGE of *P. serotina* PH isozymes. Homogeneous preparations of PH I (lane 1) and PH II (lane 2) were isolated as described in "Materials and Methods" and subjected to nondenaturing PAGE (8% gels). Proteins were detected by silver staining. This method clearly resolves PH II into dimeric (PH IIa) and monomeric (PH IIb) isozymes.

Table III. *Summary of the Purification of PH Isozymes from P. serotina Seeds*

The three PH isozymes were purified from mature black cherry seeds as described in "Materials and Methods."

Purification Step	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	mg	units[a]	units/mg	%	-fold
Crude homogenate	914	310	0.34	100	
Con A-Sepharose 4B	17.10	117	6.84	37.70	20.1
DEAE-cellulose	4.65	91.20	19.40	29.40	57.1
Reactive Red 120 agarose					
PH I	0.10	17.00	170	5.48	500
PH II	0.03	8.88	296	2.90	871

[a] One unit of enzyme activity yields 1 mmol glucose h^{-1} under standard assay conditions.

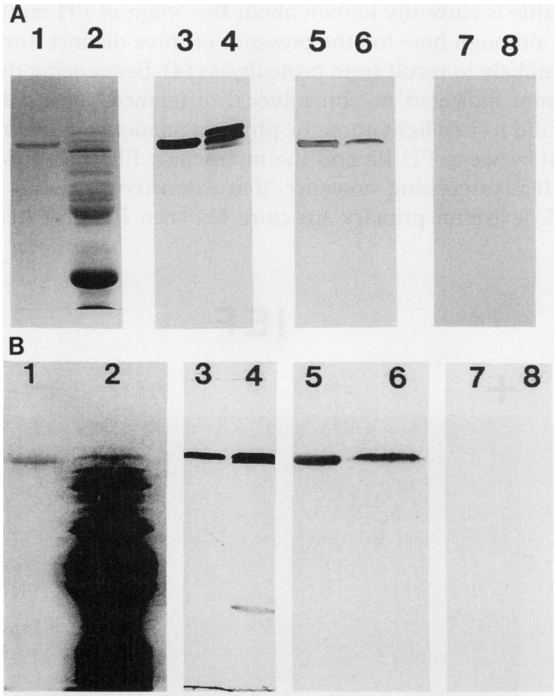

Figure 4. Effect of affinity purification on the specificity of rabbit anti-AH (A) and anti-PH (B) antisera. Total cherry seed proteins (odd-numbered lanes) and the respective homogeneous glycosidase (even-numbered lanes) were subjected to SDS-PAGE (10% gels). Proteins in lanes 1 and 2 were stained with brilliant blue G. Proteins in other lanes were electroblotted onto nitrocellulose membranes and challenged with the following preimmune sera or antisera: lanes 3 and 4, IgG-enriched rabbit antiserum, diluted 1:500 in TBS containing 5% (w/v) nonfat dry milk and 0.05% (v/v) Tween 20; lanes 5 and 6, affinity-purified IgG-enriched rabbit antiserum, diluted 1:500 as described above; lanes 7 and 8, IgG-enriched preimmune serum, diluted 1:500 as described above.

deglycosylated PH isozymes were also not completely monospecific but additionally recognized AH. Antibodies recognizing the latter hydrolase were removed by affinity chromatography and absorption on treated nitrocellulose membranes as described in "Materials and Methods." The monospecificity of the resulting antisera toward PH was confirmed by immunoblotting crude cherry homogenates on one- and two-dimensional PAGE (Figs. 4B and 7). Dot immunobinding techniques indicated that the purified antiserum recognized all PH isozymes (Fig. 6). PH I (50 ng) was recognized at an antibody dilution of 1:2000, whereas PH IIa and IIb, at equivalent levels, were recognized at even greater antibody dilutions (1:5000) (data not shown). With monospecific antibodies available that recognize all known AH and PH isozymes, the immunocytochemical localization of these glycosidases in black cherry seeds now becomes feasible.

Possible Origins of AH and PH Multiple Forms

One of the complexities of the *Prunus* cyanogenic system yet to be satisfactorily unraveled is the nature and physiological significance of the microheterogeneity shown by AH and PH. Shown also by analogous enzymes from other rosaceous stone fruits (6, 25), such multiplicity might reflect the existence of multigene families, differences in posttranslational modifications, aggregation-dissociation phenomena (8, 9, 24), or even partial proteolysis during enzyme isolation (17). Allelic variations are also possible because our enzyme source consists of seeds collected from many individuals or, at best, a single tree. In this context, the tetraploid nature of the *P. serotina* genome should be kept in mind.

Although partial proteolysis during protein isolation may lead to size heterogeneity in some cases (e.g. for pea lipoxygenase, ref. 3), several lines of evidence argue against its involvement in generating AH microheterogeneity: (a) all AH isozymes have similar molecular masses; (b) the inclusion of several protease inhibitors during extraction and purification of AH did not alter elution profiles (13); (c) N-terminal sequence analysis of the four isozymes indicated minor but significant differences that cannot easily be attributed to partial proteolysis during isolation (Fig. 8). The extensive sequence homology between isozymes may at least in part underlie the finding that polyclonal antibodies raised against

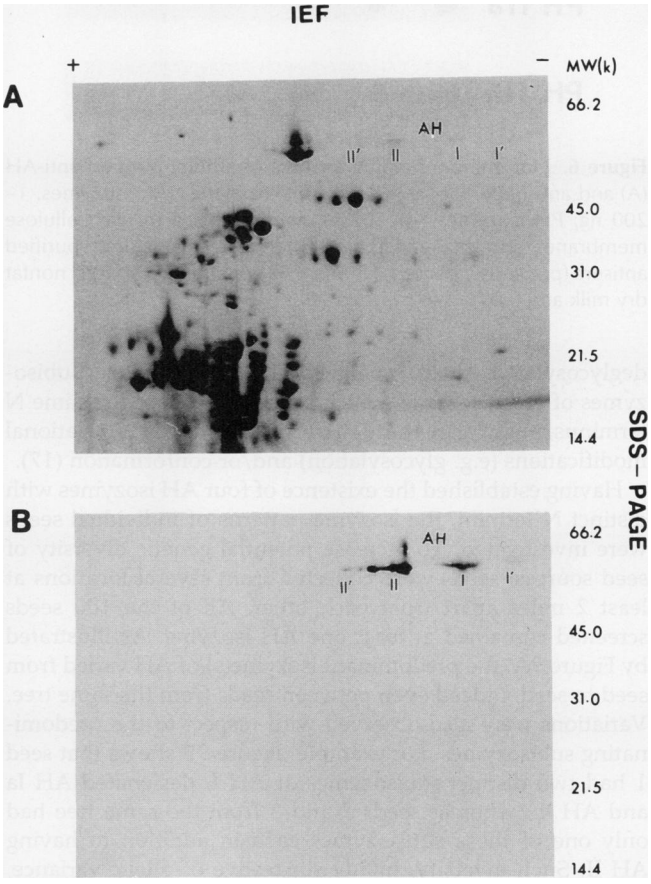

Figure 5. Two-dimensional immunoblot analysis of specificity of affinity-purified anti-AH antisera. Total cherry proteins were resolved by IEF followed by SDS-PAGE (10–15% gradient gel). Proteins were either located by brilliant blue G staining (A) or electroblotted onto nitrocellulose filters and challenged with affinity-purified anti-AH antiserum previously diluted 1:500 in TBS containing 5% (w/v) nonfat dry milk and 0.05% (v/v) Tween 20 (B).

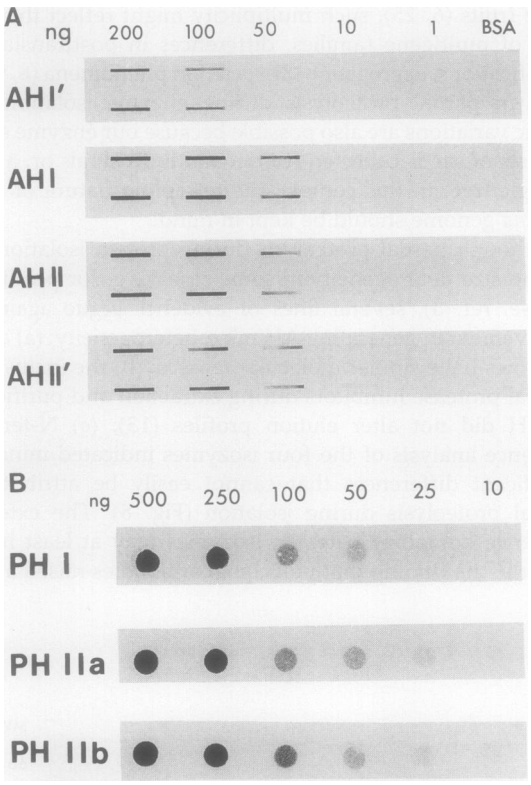

Figure 6. Dot immunobinding analysis of affinity-purified anti-AH (A) and anti-PH (B) antisera. Individual isozymes (AH isozymes, 1–200 ng; PH isozymes, 10–500 ng) were applied to nitrocellulose membranes and challenged with the respective affinity-purified antisera (previously diluted 1:500 in TBS containing 5% [w/v] nonfat dry milk and 0.05% [v/v] Tween 20).

deglycosylated AH I recognized all four isozymes. Subisozymes of each isozyme, which apparently share the same N terminus, might arise through differences in posttranslational modifications (e.g. glycosylation) and/or conformation (17).

Having established the existence of four AH isozymes with distinct N termini, the isozyme patterns of individual seeds were investigated. To increase potential genetic diversity of seed sources, seeds were collected from several locations at least 2 miles apart from each other. All of the 100 seeds screened contained at least one AH isozyme. As illustrated by Figure 9A, the predominant isozyme(s) of AH varied from seed to seed, indeed even between seeds from the same tree. Variations were also observed with respect to the predominating subisozymes. For example, Figure 9B shows that seed 1 had two distinct subisozymes of AH I, designated AH Ia and AH Ib, whereas seeds 2 and 3 from the same tree had only one of these subisozymes each in addition to having AH II. Such diversity, highly suggestive of allelic variance, explains our observation that although chromatofocusing profiles consistently suggested the presence of four AH isozymes, the relative amount of each isozyme varied from purification to purification. β-Glycosidase polymorphism is known in other species. With over 30 different alleles, maize β-glucosidase is the most polymorphic enzyme locus reported in plants (5).

Little is currently known about the origin of PH multiplicity, although here too the presence of three distinct isozymes is unlikely to result from proteolysis (14). Sequencing their N termini indicated no unequivocal differences among them, nor did it shed light upon the physical relationship that might exist between PH IIa and the monomeric PH isozymes (Fig. 8). It is interesting however, that extensive homology (53–75%) exists in primary structure between PH and AH iso-

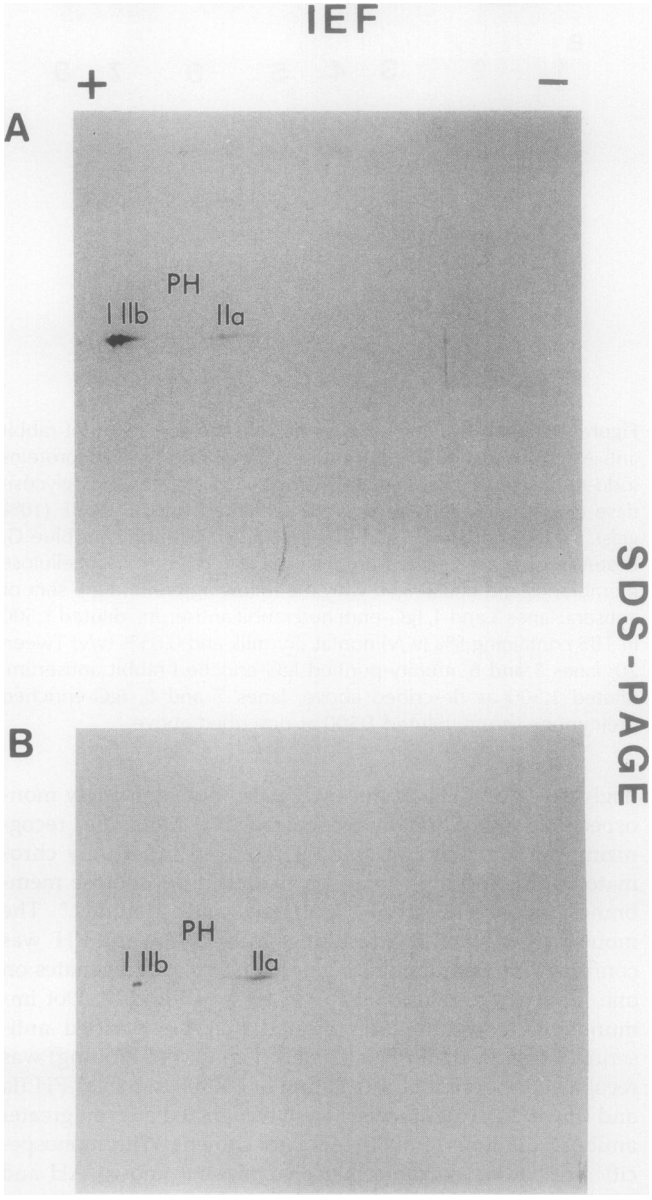

Figure 7. Two-dimensional immunoblot analysis using affinity-purified rabbit anti-PH antiserum. Total cherry seed proteins (A) or a mixture containing approximately equal amounts of the three homogeneous PH isozymes (B) were resolved by IEF (pH 3–9) followed by SDS-PAGE (10% gels). Proteins were electroblotted onto nitrocellulose membranes and challenged with affinity-purified rabbit anti-PH antiserum previously diluted 1:500 in TBS containing 5% (w/v) nonfat dry milk and 0.05% (v/v) Tween 20.

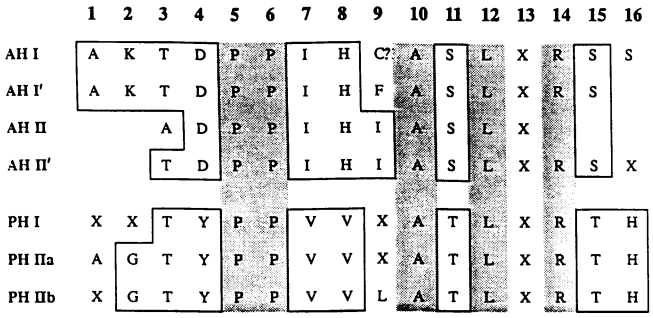

Figure 8. Comparison of the N-terminal amino acid sequences of *P. serotina* AH and PH isozymes. Amino acids are indicated by single-letter IUPAC nomenclature with the letter X denoting position of unidentified residue. Sequences have been aligned to maximize similarity. Shaded boxes show amino acids common to all seven isozymes, whereas white boxes show residues that are shared by either the AH or the PH isozymes.

zymes, including the Pro-Pro dipeptide at residues 5 and 6. It is hoped that future molecular biological approaches will shed further light on the physiological significance and evolutionary relationship of AH and PH multiple forms.

ACKNOWLEDGMENTS

The authors wish to thank Linda Anthony and Tony Nevshemal for assistance in manuscript preparation.

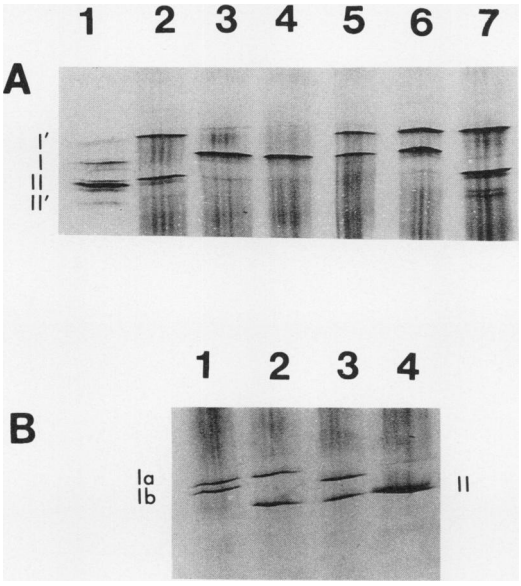

Figure 9. Heterogeneity observed in AH isozyme patterns of individual seeds. AH was extracted from individual seeds and partially purified by Con A-Sepharose chromatography before being subjected to IEF on Phastgel IEF 3/9 gels followed by silver staining. A, Variation in AH isozyme patterns. Lane 1, highly purified AH, exhibiting all four isozymes; lanes 2 and 3, lanes 4 and 5, and lanes 6 and 7 show isozyme patterns from pairs of seeds taken from three different trees located at sites at least 2 miles apart. B, Variation in subisozyme patterns. Lanes 1 to 4, isozyme patterns of four different seeds collected from a single tree.

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