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Purification and Characterization of Hydroxypyruvate Reductase from Cucumber Cotyledons[1]

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DAVID E. TITUS, DAVID HONDRED, AND WAYNE M. BECKER
Department of Botany, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Hydroxypyruvate reductase (HPR), a marker enzyme of peroxisomes, has been purified to homogeneity from cotyledons of light-grown cucumber seedlings (*Cucumis sativus* var. Improved Long Green). In addition, the peroxisomal location of both HPR and serine-glyoxylate aminotransferase has been confirmed in cucumber cotyledons. The isolation procedure involved Polymin-P precipitation, a two-step precipitation with ammonium sulfate (35 and 50% saturation), affinity chromatography on Cibacron Blue-agarose, and ion-exchange chromatography on DEAE-cellulose. HPR was purified 541-fold to a final specific activity of 525 ± 19 micromoles per minute per milligram of protein. Enzyme homogeneity was established by native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The native molecular weight was 91 to 95 kilodaltons, approximately double the apparent subunit molecular weight of 40,500 ± 1,400. With hydroxypyruvate as substrate, the pH optimum was 7.1 and K_m values were 62 ± 6 and 5.8 ± 0.7 micromolar for hydroxypyruvate and NADH, respectively. With glyoxylate as substrate, the pH optimum was 6.0, and the K_m values for glyoxylate and NADH were 5700 ± 600 and 2.9 ± 0.5 micromolar, respectively. Antibodies to HPR were raised in mice (by the ascites tumor method) and in rabbits, and their monospecificity was demonstrated by a modified Western blot immunodetection technique.

Seedlings of fat-storing species undergo a postgerminative transition from chemotrophy to phototrophy that involves a change in the metabolic role of microbodies from fat utilization (glyoxysomes) to photorespiratory glycolate metabolism (peroxisomes) (2, 4). In greening cotyledons, the increase in peroxisomal enzyme activities typically occurs at the same time as the decrease in glyoxysomal activities (4, 18), evidently in the absence of cell division (2). The mechanism underlying this changeover in microbody function has not been definitively established and has in fact generated considerable controversy (4, 6, 8, 23, 28).

To date, we have focused on the regulation of glyoxysomal enzymes in cucumber cotyledons. We have isolated several glyoxysomal enzymes (16, 20), raised antibodies to them, and used these to investigate both the dependence of enzyme appearance upon translatable mRNA levels (29) and the compartmentalization of these enzymes within glyoxysomes (3, 20). To extend such studies to the biogenesis of peroxisomes and thus to the mechanism underlying the transition from glyoxysomal to peroxisomal function, one or more peroxisomal marker enzymes need to be purified and used to prepare monospecific antibodies. One such enzyme is NADH-dependent HPR,[2] which was first purified from tobacco leaves by Zelitch (30) and was later characterized by Kohn and Warren (14).

The present paper describes the purification and characterization of HPR from cucumber cotyledons and confirms the peroxisomal location of the enzyme. Monospecific antibodies to HPR have been raised in both rabbits and mice. These antibodies will be used to assay for both peroxisomal enzyme appearance and compartmentalization during the transition in microbody function. A preliminary report of this work has been published in abstract form (26).

MATERIALS AND METHODS

Sources. All materials were obtained as described previously (16) except for the following. Acrylamide, Cibacron Blue F3GA-agarose, and agarose powder were purchased from Bio-Rad. Ultrapure ammonium sulfate was a product of Schwarz/Mann. Alkaline phosphatase-coupled affinity-purified goat antisera against rabbit and mouse immunoglobulins were obtained from Kierkegaard and Perry Laboratories, Inc. Most other reagents were purchased from Sigma Biochemicals. Nitrocellulose sheets were Millipore HA-type (0.45-μm pore size). Female ICR mice came from Harlan-Sprague-Dawley. Sarcoma cells were a generous gift from Biotec Corporation, Madison, WI.

Buffers and Solutions. Buffer pH values were determined at 25°C. GB contained 87.5 mM Tris-HCl (pH 7.7), 1.75 mM EDTA, 7 mM $MgCl_2$, and 10 mM DTT. WB was 10 mM Tris-HCl (pH 7.7) and 1 mM EDTA. Recycling buffer for the Cibacron Blue column contained 6 M guanidine-HCl and 1.2 M NaCl in WB. PBS contained 10 mM sodium phosphate (pH 7.2) and 150 mM NaCl. Incubation and washing steps for the immunodetection assay were performed in TBS which contained 10 mM Tris-HCl (pH 7.4), 0.9% (w/v) NaCl, 0.1% (w/v) BSA, and 0.02% (w/v) NaN_3. Washing buffers contained in addition 1% (w/v) BSA (TBS + BSA), 0.05% (v/v) NP-40 (TBS + N), or 0.05% NP-40 plus 0.05% (w/v) SDS (TBS + NS). NADH was prepared as a stock solution of 88.8 mM NADH, 80 mM Na_2CO_3, and 20 mM $NaHCO_3$ and stored at −60°C in 0.1-ml aliquots. The commercial Polymin-P (50%) was diluted to a 10% (v/v) stock solution and titrated to pH 7.7 with HCl.

HPR Assay. HPR activity was assayed using a procedure slightly modified from that described by Kohn and Warren (14).

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[2] Abbreviations: HPR, hydroxypyruvate reductase; DMSO, dimethyl sulfoxide; GAM-AP, alkaline phosphatase-coupled goat anti-mouse IgG serum; GAR-AP, alkaline phosphatase-coupled goat anti-rabbit IgG serum; GB, grinding buffer; NBT, nitro blue tetrazolium; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; Polymin-P, polyethyleneimine; SGAT, serine-glyoxylate aminotransferase; TBS, Tris-HCl/NaCl/BSA/NaN_3 buffer; WB, column washing buffer.

The assay mixture contained 60 mM K-phosphate (pH 6.2), 0.016% (v/v) Triton X-100, 0.167 mM NADH, and 0.5 mM lithium hydroxypyruvate in a volume of 1.0 ml, plus an additional 10 to 50 μl of enzyme preparation diluted if necessary with distilled, deionized H_2O. Purified enzyme solutions diluted in this manner and stored on ice were stable for more than 4 h (data not shown). NADH oxidation was monitored as the change in absorption at 340 nm at 25°C. One unit of HPR activity is defined as that quantity of enzyme which will catalyze the oxidation of 1.0 μmol of NADH/min in a standard assay.

***In Situ* HPR Assay.** The *in situ* assay for HPR in native polyacrylamide gels was adapted from an assay for homoserine dehydrogenase described by Matthews *et al.* (17). The reaction is performed in the direction of glycerate oxidation and results in the appearance of reduced NBT in the gel as a purple precipitate. Immediately after electrophoresis, the native gel was placed in the *in situ* assay solution and stained for 20 to 30 min in a light-proof box. The *in situ* assay solution contained 200 mM Tris (pH 8.9), 58 mM DL-glycerate, 0.33 mM NBT, 2.2 mM NAD, and 0.081 mM PMS. The reaction was stopped by addition of 7.5% acetic acid, which also removed the yellow background color. A control staining in the absence of glycerate was also performed.

Other Assays. Published procedures were followed when assaying sucrose gradient fractions for catalase (16), Cyt *c* oxidase (24), and Chl (1). The SGAT assay was performed as described by Rehfeld and Tolbert (19) except that the final assay mix contained 70 mM Hepes (pH 8.2), 0.3 mM NADH, 0.1 mM pyridoxal-5-P, 4 mM glyoxylate, 20 mM L-serine, and 0.03 unit/ml spinach glyoxylate reductase.

Protein was determined with the assay of Bradford (7) as modified by Spector (25). Column effluents were monitored photometrically (at 280 nm) for protein. For sucrose gradient fractions and samples containing glycerol, protein was assayed by a modified Lowry procedure (5) with ovalbumin as the standard. The conductivity of column fractions was determined with a Radiometer Copenhagen conductivity meter. The density of sucrose gradient fractions was determined from measurements of their refractive indices.

Subcellular Localization of HPR. To demonstrate the peroxisomal location of HPR, sucrose gradient centrifugation of a cotyledonary homogenate (*Cucumis sativus* var. Improved Long Green) was carried out in a vertical rotor essentially as described by Donaldson (11). Seven-d, light-grown cucumber cotyledons (20 g) were picked onto ice and chopped 10 min with razor blades in 20 ml of 50 mM Tricine (pH 7.7), 1 mM EDTA, 10 mM DTT, and 20% (w/w) sucrose. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth, and then centrifuged at 500*g* for 10 min in a swinging bucket rotor. A 10.5-ml aliquot of the supernatant was layered onto each of two 22-ml gradients consisting of 27 to 60% (w/w) sucrose in 50 mM Tricine (pH 7.7) and 1 mM EDTA, overlaid with three ml of 24% (w/w) sucrose in the same buffer. The gradients were centrifuged for 80 min at 20,000 rpm (40,300g_{max}) in a DuPont/Sorvall SV-288 vertical rotor using the slow acceleration/deceleration mode of the centrifuge. All procedures were performed at 0 to 5°C.

Enzyme Isolation. Cucumber seedlings were grown in continuous light at 25°C for 7 d. Cotyledons were harvested onto ice, chopped with a razor blade, and homogenized in 2 volumes of GB using a Polytron homogenizer with a PT 20 probe. All subsequent operations were carried out on ice at 4°C. The homogenate was squeezed through three layers of cheesecloth, filtered through Miracloth, and centrifuged at 27,500*g* for 15 min to remove starch and insoluble cell debris. Polymin-P was added to the resulting supernatant (S-1) to a final concentration of 0.1%. After stirring for 15 min, the solution was centrifuged at 20,000*g* for 10 min. To this supernatant (S-2), ammonium sulfate was added slowly with stirring to a final concentration of 19.4 g/100 ml (approximately 35% saturation at 0°C). This solution was stirred for 30 min, then centrifuged at 20,000*g* for 10 min. Additional ammonium sulfate (8.7 g/100 ml; about 50% saturation) was added to the supernatant (S-3), followed by stirring for 30 min and by centrifugation at 20,000*g* for 10 min. The pellet (P-4) was resuspended in WB and dialyzed against three changes of WB.

The dialyzed sample was centrifuged at 20,000*g* for 10 min and the supernatant was loaded onto a Cibacron Blue F3GA-agarose column (11-ml bed volume) which had been equilibrated with WB. The loaded column was washed with 5 column volumes of WB, followed by 5 column volumes of 0.1 M NaCl in WB. HPR was then eluted with a linear gradient (0–0.5 mM) of NADH in 200 ml of WB. The fractions with peak HPR activity (298–335 ml elution volume) were pooled and loaded directly onto a DEAE-cellulose column (5.3-ml bed volume) equilibrated with WB. The column was washed with 5 column volumes of WB, and a linear gradient (0–200 mM) of NaCl in 200 ml of WB was used to elute purified HPR. Fractions with peak HPR activity were stored in 50% glycerol at −20°C. For the purpose of raising antibodies, some HPR was further purified by electrophoresis in SDS gels. HPR was visualized and eluted from the gels by the method of Hager and Burgess (12).

Kinetic Constants. Values for K_m were obtained with a modified enzyme assay containing 60 mM K-phosphate, 0.16% (v/v) Triton X-100, variable concentrations of NADH (5–60 μM), variable concentrations of hydroxypyruvate (25–500 μM), or glyoxylate (1–7 mM), and 50 to 60 μl of enzyme solution in a final volume of 3.0 ml. The assay pH was either 7.1 (with hydroxypyruvate) or 6.0

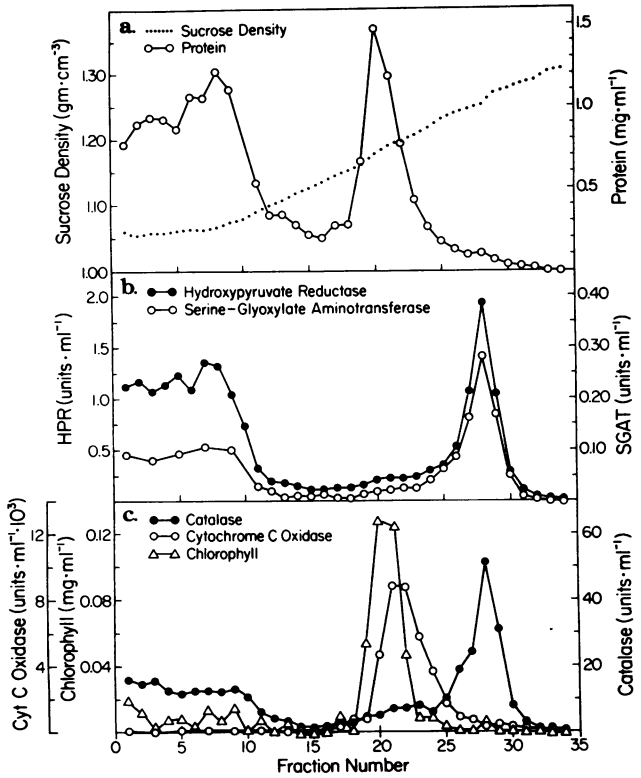

FIG. 1. Sucrose gradient fractionation of a cotyledonary homogenate of 7-d-old light-grown cucumber seedlings. A 10.5-ml aliquot of 500*g* supernatant was layered onto each of two identical 25-ml gradients prepared as described in the text. After centrifugation for 80 min at 40,300*g* in a vertical rotor, 1.05-ml fractions were collected and assayed for refractive index, protein content, Chl, and enzyme activities. The symbol representing each assay is identified within the corresponding portion of the figure.

Table I. *Summary of Hydroxypyruvate Reductase Purification*

The starting material was 333 g cotyledons.

Fraction	Volume	Enzyme Activity	Protein	Specific Activity	Purification	Yield
	ml	*units*	*mg*	*units/mg*	*-fold*	*%*
Filtrate	880	3942	4048	0.97	1.0	100
S-1	479	3162	2157	1.47	1.5	80
S-2	735	2765	456	6.06	6	70
S-3	790	2734	348	7.86	8	69
P-4	78	2715	150	18.1	19	69
Blue pool[a]	60	1357				34
DEAE-peak[b]	20	866	1.65	525 ± 19	541	22

[a] Pooled HPR fractions from Cibacron blue-agarose.

[b] These values are summations of data for the four peak fractions (66–69) from DEAE-cellulose.

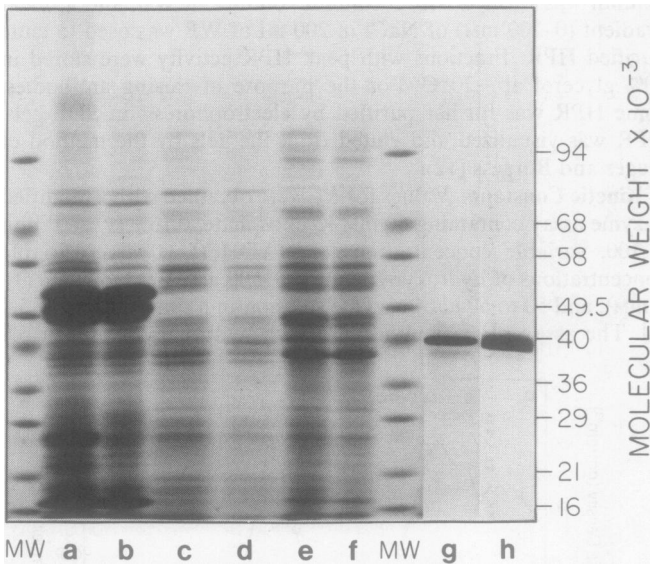

FIG. 2. Progress of HPR purification as assayed on SDS-polyacrylamide slab gels. Aliquots from each stage of purification were subjected to electrophoresis on 7.5 to 15% gradient slab gels. The lanes contained (a) 100 μg of filtrate, (b) 49 μg of S-1, (c) 55 μg of S-2, (d) 24 μg of S-3, (e) 39 μg of P-4, (f) 25 μg of the dialyzed P-4, (g) approximately 4 μg of the Cibacron blue-agarose peak fraction, and (h) approximately 4 μg of the DEAE-cellulose peak fraction. Mol wt markers are included where indicated, with 2 μg of each marker loaded/lane.

(with glyoxylate). The reaction was started by the rapid addition of hydroxypyruvate or glyoxylate. Initial 1-min velocities were determined, and K_m values were calculated using two computer programs (SEQUEN and PING-PONG) kindly provided by W. W. Cleland (10). The enzyme preparation used to determine the pH optima and kinetic parameters of HPR was obtained by an earlier procedure in which the DEAE-cellulose step preceded chromatography on Cibacron Blue-agarose. The enzyme isolated in this way was electrophoretically identical to that prepared by the procedure described here.

Electrophoresis Procedures. SDS-PAGE (15) and native-PAGE were performed using 1.5-mm thick slab gels. Except where otherwise noted, SDS gels were cast with a linear 7.5 to 15% gradient of acrylamide, while 4% acrylamide gels were generally used for native PAGE. The acrylamide:bisacrylamide ratio was 150:1 for SDS gels and 37.5:1 for native gels. All solutions used for native gels were identical to those for SDS gels except that they contained no SDS or 2-mercaptoethanol. Gels were electrophoresed until the bromophenol blue dye front reached the end of the gel.

Molecular Weight Determination. The apparent subunit mol wt of HPR was calculated by interpolation from a plot of mobility on SDS-polyacrylamide gels *versus* log mol wt. The native mol wt of HPR was determined both by gel filtration chromatography on a calibrated Sephacryl S-200 column and by the native gel technique of Hedrick and Smith (13). In the native gel approach, the effect of acrylamide concentration of the gel on electrophoretic mobility of a given protein is used to distinguish charge and size effects on the mobility of related bands. The substitution of slab gels for the tube gels used by Hedrick and Smith resulted in a considerable saving of effort.

Preparation of Antisera. Antiserum against cucumber HPR was raised in a 2-kg rabbit by subcutaneous injection of 100 μg of purified enzyme in 1.4 ml of 50% (v/v) complete Freund's adjuvant followed 4 weeks later by intramuscular injection of another

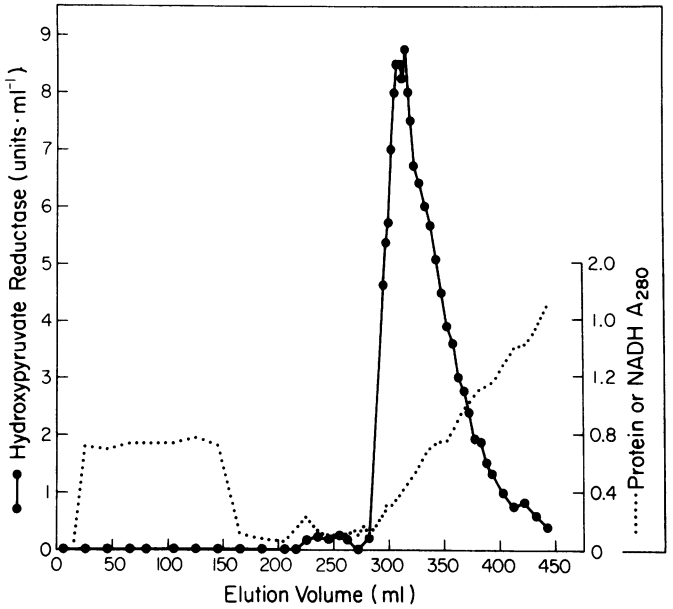

FIG. 3. Affinity chromatography of HPR on Cibacron blue-agarose. About half of the HPR-enriched dialysate was diluted (141-ml final volume) and loaded onto a Cibacron blue-agarose column (3.9 × 1.9 cm) equilibrated with WB. The column was washed with 55 ml of WB followed by 55 ml of WB containing 100 mM NaCl. Fraction volumes were then reduced from 5.0 to 2.5 ml and the column was eluted at a flow rate of 43 ml/h with a linear NADH gradient (0–0.5 mM in 200 ml of WB). The effluent was monitored for protein (before elution) or NADH (during elution) at A_{280}. The peak fractions (298–335-ml elution volume) were pooled and combined with peak fractions resulting from Cibacron blue-agarose chromatography of the other portion of the dialysate (column not shown).

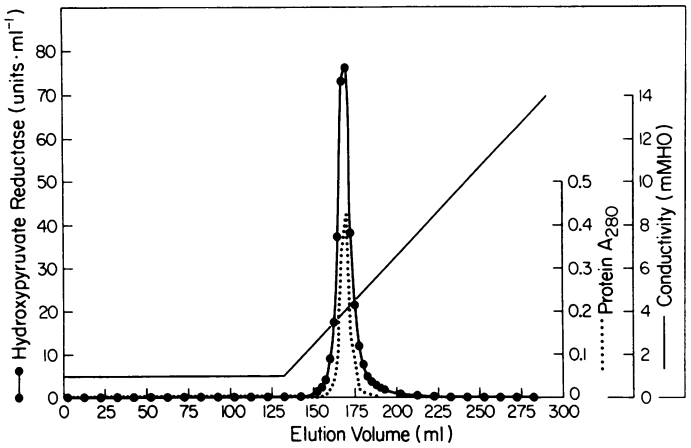

FIG. 4. Purification of HPR by DEAE-cellulose ion-exchange chromatography. The pooled HPR fractions (60 ml) from the Cibacron blue-agarose column of Figure 3 were loaded onto a DEAE-cellulose column (6.7 × 1.0 cm) equilibrated with WB. The column was washed with 45 ml of WB and then eluted with a linear NADH gradient (0–200 mM) in 200 ml of WB at a flow rate of 33 ml/h. Fractions of 2.5 ml were collected and assayed for HPR. Fractions 66 to 69 were stored at −20°C in 50% glycerol and subsequently used for enzyme characterization and for raising antibodies.

100 μg in 50% (v/v) incomplete adjuvant. The rabbit was bled at 10, 17, and 24 d after the second injection. Later, additional serum was obtained with a booster injection of 100 μg of HPR in 50% (v/v) incomplete adjuvant followed by bleedings as described above. Antiserum against spinach HPR was raised in the same fashion, except that the antigen was purchased from Sigma and purified by elution from SDS-polyacrylamide gels (12). Mouse antibodies were obtained by the ascites tumor method (21).

Immunodetection of Proteins on Polyacrylamide Gels. For the immunodetection assay, samples were electrophoresed on 12% SDS gels or 4% native gels. Transfer of proteins to nitrocellulose (Western blotting, Ref. 9) was performed in a Bio-Rad Trans-Blot apparatus at 200 or 250 mamp (constant current) for 12 to 20 h. Transfer buffer contained 12.4 mM Tris (pH 8.3), 96 mM glycine, and 20% (v/v) methanol.

Following electrophoretic transfer, the nitrocellulose sheet was incubated in TBS + BSA at 37°C for 1 h, and then transferred to a heat-sealed plastic bag containing rabbit serum or mouse ascitic fluid which had been diluted into 5 ml of TBS + BSA. All subsequent steps were performed at room temperature. After incubation for 1 to 2 h, the sheet was washed three times (20 min each) with 40 ml of TBS + N and then briefly rinsed with 40 ml of TBS. To detect bound HPR-specific immunoglobulins, the sheet was incubated for 1 h in a plastic bag containing 5 ml of TBS + BSA plus diluted alkaline phosphatase-conjugated goat serum directed against either rabbit or mouse IgG (GAR-AP or GAM-AP, respectively). After this incubation, the sheet was washed three times (20 min each) with 40 ml of TBS + NS and then briefly rinsed with 40 ml of TBS.

Alkaline phosphatase activity coupled to the goat antibodies was detected with the technique of D. A. Knecht and R. L. Dimond (manuscript in preparation). An agar slab containing 8.8 mg of 5-bromo-4-chloro-3-indolyl phosphate, initially dissolved in 25 μl of DMSO, 5.3 ml of 2.5 M Tris (pH 9.5), and 12.3 ml of 3% agar (Difco Bacto-agar) was cast on a glass plate (11 × 16 cm). The washed nitrocellulose sheets were laid face down on the agar slab and the resultant sandwich was enclosed in plastic wrap and allowed to react at room temperature until the blue staining reached the desired intensity (1–18 h). Both the nitrocellulose sheet and the agar slab were gently washed in H_2O and then dried for storage.

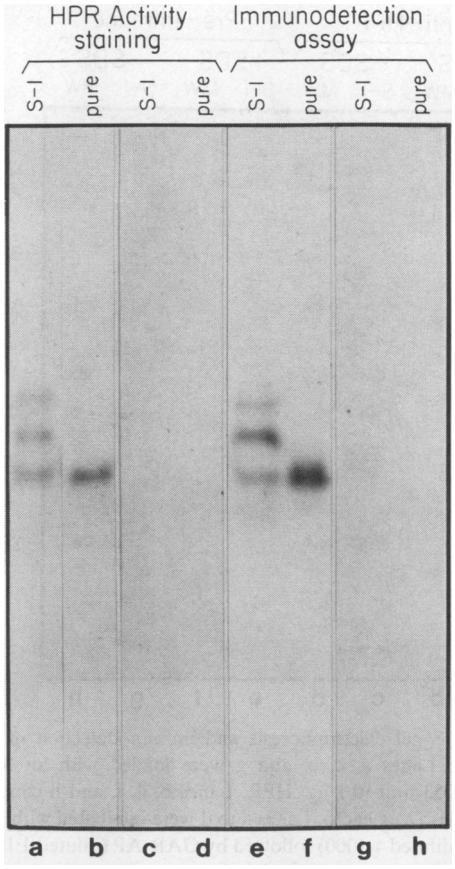

FIG. 5. Nondenaturing gel electrophoresis of HPR from crude or purified fractions. Lanes a, c, e, and g were loaded with an S-1 fraction containing 0.053 unit (0.1 μg) HPR. Lanes b and d contain 0.5 μg of purified HPR, while lanes f and h contain 0.1 μg of purified HPR. Lanes a to d: *in situ* HPR activity staining. The positive staining reaction (lanes a and b) is contrasted with the control reaction lacking glycerate (lanes c and d). Lanes e to h: gel immunodetection assay. Lanes e and f were incubated with rabbit anti-HPR serum (diluted 1:1000) followed by GAR-AP (diluted 1:1000). In lanes g and h, pre-immune rabbit serum (diluted 1:1000) was substituted for the anti-HPR serum. Details of the incubation steps and enzyme-mediated visualization procedure are presented in the text.

Table II. *Properties of Hydroxypyruvate Reductase*

Native mol wt	91,000–95,000
Subunit mol wt	40,500 ± 1,400
Specific activity (with hydroxypyruvate)	525 ± 19 units/mg
pH optimum with hydroxypyruvate	6.9–7.3
pH optimum with glyoxylate	5.8–6.2
K_m for hydroxypyruvate (pH 7.1)	62 ± 6 μM
K_m for glyoxylate (pH 6.0)	5700 ± 600 μM
K_m for NADH (pH 7.1)	5.8 ± 0.7 μM
K_m for NADH (pH 6.0)	2.9 ± 0.5 μM

RESULTS

Subcellular Fractionation. The subcellular location of HPR was verified by fractionation on sucrose gradients of cotyledonary homogenates from 7-d light-grown seedlings (Fig. 1). HPR and SGAT banded with catalase, a peroxisomal marker, at a mean density of 1.26 g/cm^3, confirming the peroxisomal location of both enzymes (Fig. 1b). The Chl peak (at 1.18 g/cm^3) and the Cyt *c* oxidase peak (at 1.19 g/cm^3) identify broken chloroplasts and mitochondria, respectively (Fig. 1c). Though poorly resolved from

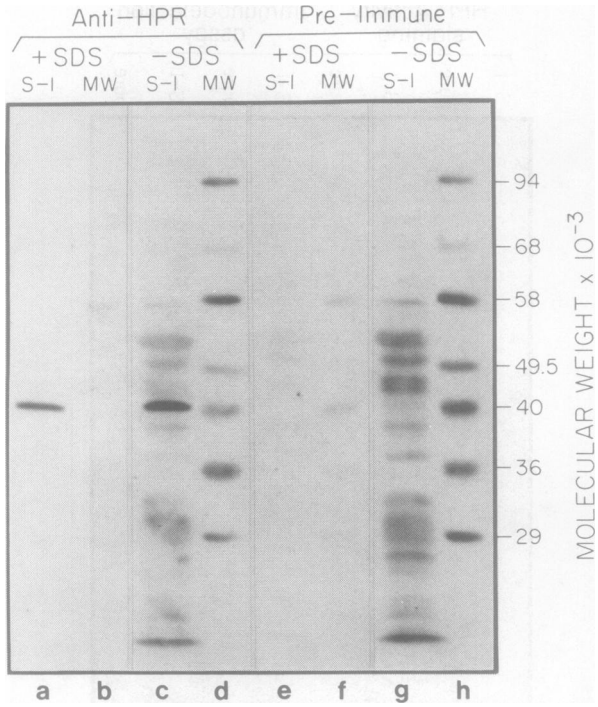

FIG. 6. SDS gel electrophoresis and immunodetection of HPR with rabbit serum. Lanes a, c, e, and g were loaded with an S-1 fraction containing 0.053 unit (0.1 μg) HPR. Lanes b, d, f, and h contain mol wt marker proteins (2 μg each). Lanes a to d were incubated with rabbit anti-HPR serum (diluted 1:1000) followed by GAR-AP (diluted 1:1000). Lanes e to h are control lanes, identical to lanes a to d except that rabbit pre-immune serum (diluted 1:1000) was substituted for the anti-HPR serum. The first two lanes in each group (a and b, e and f) depict results obtained with our standard immunodetection procedure which includes 0.05% SDS in the final series of washing steps. The second two lanes in each group (c and d, g and h) illustrate the nonspecific detection of protein bands observed when SDS is omitted from the final washes. Details of the standard immunodetection procedure are described in the text.

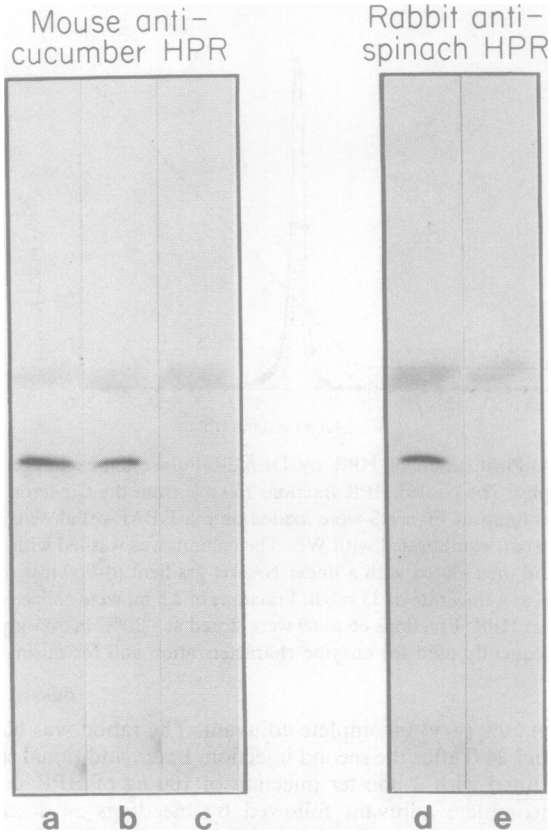

FIG. 7. Immunodetection of HPR on SDS gels with mouse ascitic fluids or rabbit anti-spinach HPR serum. All lanes were loaded with an S-1 fraction containing 0.053 unit (0.1 μg) HPR. Lanes a to c: the primary antibody incubation step was performed with ascitic fluid (diluted 1:500) from a mouse injected with either native, purified HPR (lane a) or denatured HPR eluted from an SDS gel (lane b). The control staining (lane c) was performed with nonimmune fluid (diluted 1:500) obtained from a third mouse. The secondary antibody incubation for all three lanes (a, b, c) contained GAM-AP at a 1:1000 dilution. Lanes d and e: the primary antibody incubation contained either serum (diluted 1:200) from a rabbit injected with denatured HPR eluted from an SDS gel (lane d) or pre-immune serum (diluted 1:200) from the same rabbit (lane e). The secondary antibody incubation for both lanes contained GAR-AP at a 1:1000 dilution. Details of the immunodetection procedure are described in the text.

one another, these latter bands were clearly separated from the peroxisomal band.

HPR Purification. The purification of HPR is summarized in Table I and Figure 2. Isolation was begun by homogenizing 333 g (fresh weight) of cucumber cotyledons. The addition of Polymin-P precipitated 79% of the S-1 protein, including virtually all of the ribulose bisphosphate carboxylase (Fig. 2, lanes b and c). After the ammonium sulfate precipitation steps, 69% of the starting HPR activity was present in the resuspended pellet, with a specific activity of 18.1 units/mg. The first four steps therefore resulted in an 18.7-fold purification.

Cibacron Blue-agarose affinity chromatography is illustrated in Figure 3. The HPR activity peak eluted at a concentration of approximately 0.1 mM NADH, as determined by *A* at 280 nm. NADH absorbs strongly at 280 nm, so this wavelength was used to monitor both protein concentration (before applying the NADH elution gradient) and NADH concentration (during the gradient elution). The absence of accurate protein assays at this step precluded calculation of a specific activity, but SDS-PAGE of the peak fraction (Fig. 2, lane g) revealed a single major band. Thus, HPR was nearly pure after a single affinity chromatography step.

The DEAE-cellulose elution profile is shown in Figure 4. The four peak fractions eluted at about 52 mM NaCl. The overall specific activity of the four peak fractions (66–69) was 525 ± 19 units/mg. HPR was therefore purified 541-fold from the starting filtrate.

Criteria of Homogeneity. Isolated HPR was shown to be homogeneous by the presence of a single band at 40.5 kD on a denaturing gel (Fig. 2, lane h). A single major Coomassie blue-staining band was also seen on nondenaturing gels (not shown). Figure 5, lane b, depicts an identical sample stained *in situ* for HPR activity, confirming that the major protein band has the expected enzymatic activity. The four peak fractions (66–69) from the DEAE column were also analyzed individually on SDS-polyacrylamide gels; in each case, the single major band was greater than 96% pure (data not shown).

Enzyme Properties. The properties of HPR are summarized in Table II. The native mol wt of HPR in a crude supernatant was determined to be 91 ± 8 kD (K_{av} = 0.26) by chromatography on Sephacryl S-200. The native gel technique of Hedrick and Smith (13) yielded similar values: approximately 95 and 92 kD for the native mol wt of HPR in a crude supernatant and a purified sample, respectively. The *in situ*-staining assay on nondenaturing gels routinely reveals three bands of HPR activity (94.9, 94.5, and 95.0 kD) in samples of crude supernatant (Fig. 5, lane a). The two more slowly migrating bands are lost over the course of the enzyme purification such that only the most rapidly migrating

band remains in the final purified fraction (Fig. 5, lane b).

The pH optimum was 6.9 to 7.3 with hydroxypyruvate, but 5.8 to 6.2 with glyoxylate. Michaelis constants were determined for HPR at these pH optima. At pH 7.1, K_m values were 62 ± 6 and 5.8 ± 0.7 μM for hydroxypyruvate and NADH, respectively. At pH 6.0, K_m values were 5700 ± 600 and 2.9 ± 0.5 μM for glyoxylate and NADH, respectively. HPR appears to have a nearly 100-fold greater affinity for hydroxypyruvate than for glyoxylate, while the affinity of HPR for NADH is high in both cases.

Immunodetection of HPR. Figure 6 illustrates the immunodetection assay which was used to evaluate antiserum specificity. Lanes a and e show SDS gel profiles of crude supernatant samples reacted with anti-HPR or pre-immune rabbit serum, respectively. A single band is visible in lane a, representing about 50 ng of HPR protein. Using a 1:1000 dilution of rabbit anti-HPR serum, bands containing as little as 10 ng HPR could also be detected (data not shown). Lanes c, d, g, and h are identical to lanes a, b, e, and f, respectively, except that SDS was omitted from the washing buffers after the incubation with GAR-AP. The omission of SDS resulted in a significant staining of nonantigenic bands.

The reactions of mouse immune and nonimmune ascitic fluids with crude supernatant samples are shown in Figure 7. Either native HPR (lane a) or denatured, gel-eluted HPR (lane b) was used as the immunogen for these mice. The anti-HPR fluids detected a single predominant band of 40.5 kD which is not seen in the nonimmune lane (lane c). Also shown in Figure 7 is the cross-reactivity of a rabbit serum raised against spinach HPR with a single cucumber polypeptide, presumably the HPR subunit.

The immunodetection assay was also used for nondenaturing gels. In this case, rabbit anti-HPR serum reacted with the three HPR bands which were initially detected by the *in situ* activity staining (Fig. 5, lanes a and e). The three bands are therefore cross-reactive. The pre-immune control lanes (g and h) are blank, as are the 'minus glycerate' control lanes (c and d) for the *in situ* activity staining. In both the activity staining (lane b) and the immunodetection assay (lane f), purified HPR is seen to co-migrate with the fastest of the three HPR bands found in the crude supernatant.

DISCUSSION

HPR and SGAT have been localized to peroxisomes in a variety of plant tissues (4). To extend the use of HPR as a peroxisomal marker enzyme to cucumber cotyledons, we have demonstrated the peroxisomal location of HPR in this tissue. Cucumber HPR also exhibits the dramatic light-dependent increase in activity characteristic of peroxisomal enzymes (22) (data not shown). Noguchi and Fujiwara (18) have recently demonstrated the presence of SGAT in peroxisomes of cucumber cotyledons. The work presented here confirms this finding.

HPR was isolated from cotyledons of 7-d light-grown cucumber seedlings and purified 541-fold to a final specific activity of 525 units/mg. The values of 91 to 95 kD for the native mol wt of cucumber HPR and 40.5 ± 1.4 kD for the mol wt of the single subunit band suggest that this is a dimer with two identical subunits. Cucumber HPR is quite similar to the spinach HPR characterized by Kohn and Warren (14). The spinach enzyme is also a dimer (97.5 kD) composed of two subunits of 46.5 kD each. Cucumber HPR exhibits a pH optimum with hydroxypyruvate that is close to neutrality (pH 6.9–7.3) while the spinach HPR has a more acidic pH optimum (pH 6.1–6.6) with this substrate. Both enzymes have a much greater affinity (lower K_m) for hydroxypyruvate than for glyoxylate: a 92-fold difference for cucumber HPR and a 1000-fold difference for spinach HPR. Tolbert (27) regards this as evidence against a role for HPR in glyoxylate reduction *in vitro*. It is likely that HPR from cucumber and spinach have some common antigenic determinants, since anti-spinach HPR serum cross-reacts with the cucumber enzyme (Fig. 7, lanes d and e).

The gel immunodetection technique is a convenient assay of antiserum specificity. Both the rabbit and mouse antibodies detect a single band at 40.5 kD. This confirms the homogeneity of the purified HPR antigen and demonstrates the usefulness of our antibodies as monospecific probes for HPR in future experiments.

In the immunodetection assay, the addition of SDS to the wash buffers used after the incubation with GAR-AP was found to decrease markedly the staining of nonantigenic protein bands. If this nonspecific staining were confined to cucumber proteins present in the crude supernatant, it might be argued that the SDS was simply masking serum heterospecificity. However, mol wt marker proteins from a variety of organisms are also stained in the absence of SDS (Fig. 6: compare lanes b and f with lanes d and h). Thus, the nonspecific staining appears to be artifactual and the use of SDS in the final washing buffer seems justifiable. In our experience, mouse ascitic fluid is more likely than rabbit serum to yield nonspecific detection of bands, particularly if the ascitic fluid is pooled from multiple tappings of a given mouse.

Serum obtained from the rabbit injected with gel-eluted HPR also reacted nonspecifically with especially prominent bands. This is illustrated in Figure 7, in which the large subunit of ribulose bisphosphate carboxylase is faintly visible in both immune and nonimmune lanes. The large amount of this polypeptide in the S-1 fraction is evident from Figure 2, lane b, which was loaded with an amount of S-1 comparable to the loadings in Figure 7.

Activity staining for HPR in gel profiles of the S-1 fraction consistently revealed three bands (Fig. 5, lane a). The native mol wt for these bands were 94.9, 94.5, and 95.0 kD for the upper, middle, and lower bands, respectively. Given the limited accuracy of the Hedrick and Smith technique (13) used for this purpose, a value of 95 kD seems appropriate for all three bands. Thus, it is likely that these multiple activity bands are resolved by virtue of differences in charge rather than size.

The significance of the three HPR bands in the S-1 fraction is unclear. They may be artifacts of our extraction procedures and may result from minor proteolysis or other modifications which eventually yield a uniform population of the fastest migrating band.

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