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Phytochrome Immunoaffinity Purification[1]

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ROBERT E. HUNT[2] AND LEE H. PRATT[3]
Department of Biology, Vanderbilt University, Nashville, Tennessee 37235

ABSTRACT

We have developed a phytochrome immunoaffinity purification procedure that yields undegraded oat (*Avena sativa* L., cv. Garry) phytochrome of greater than 98% purity within 2 hours when starting with a brushite-purified preparation. Immunoaffinity-purified phytochrome, except for its greater purity, is indistinguishable from conventionally purified phytochrome by gel exclusion chromatography, isoelectric focusing, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We have also used the immunoaffinity technique to purify phytochrome from crude oat extracts, and from brushite-purified pea (*Pisum sativum* L., cv. Alaska) and rye (*Secale cereale* L., cv. Balbo) preparations.

Purification techniques for undegraded oat phytochrome are usually based on modifications of the Rice *et al.* (16) procedure for undegraded rye phytochrome (*cf.* refs. 11 and 12 for discussions). The presence of endogenous oat proteases (4), which partially co-purify with phytochrome and generate a stable, 60,000-dalton, phytochrome degradation product (16), requires rapid manipulation during the 3- to 5-day purification procedure to minimize the action of these proteases. While both Cundiff (2) and Roux *et al.* (18) have reported homogeneous preparations of undegraded phytochrome from oats, as judged by SDS PAGE,[4] routine purifications using these procedures normally yield a product that is only about 30 to 70% homogeneous. Clearly, a simple purification technique that yields homogeneous, undegraded phytochrome is desirable.

We report here a new method for the purification of undegraded phytochrome using agarose-immobilized antiphytochrome IgGs. This simple and rapid procedure yields oat phytochrome that is greater than 98% homogeneous as judged as SDS PAGE and is indistinguishable from conventionally purified phytochrome using several physicochemical assays.

MATERIALS AND METHODS

Plants. Oats (*Avena sativa* L., cv. Garry), rye (*Secale cereale* L., cv. Balbo), and pea (*Pisum sativum* L., cv. Alaska) were grown on moist cellulose pads at near saturating humidity in darkness at 25 C. Shoots were harvested as previously described (10, 13) and stored at −20 C until extracted. All handling of plants and phytochrome preparations was done under dim green safelights (10).

Conventional Phytochrome Preparations. Degraded phytochrome was purified to a specific absorbance ratio (SAR = A_{667}/A_{280}) of 1.20 to 1.30 as previously described (5). One unit of degraded phytochrome is that quantity which in 1 ml gives $A^{1\,cm}_{667}$ = 1.0 after saturating far red irradiation (11, 12).

Degraded phytochrome was coupled to cyanogen bromide-activated agarose (Pharmacia, 74301 or Sigma, C-9142; capacity 8 to 12 mg protein/ml agarose) according to manufacturer's instructions (9). Phytochrome, in 0.1 M Na-phosphate (pH 7.8), was made 0.5 M with solid NaCl and pH was adjusted to 8.3 with 1 N NaOH. CNBr-activated agarose (120 mg/unit phytochrome) was washed extensively with 1 mM HCl and then gently mixed with phytochrome (pH 8.3) overnight at 4 C. Agarose beads were then pelleted by a 2-min, 1,500*g* centrifugation, the supernatant was decanted, and 0.1 M monoethanolamine (pH 9.0) (4 ml/ml agarose) was added and incubated with the beads overnight at 4 C with gentle mixing. Agarose-immobilized phytochrome was layered over a Sephadex G-25 column (10 ml G-25/ml agarose) and washed with about 30 ml of 25 mM MOPS-Tris (prepared by mixing 25 mM MOPS with 25 mM Tris to obtain desired pH), 5 mM EDTA (pH 7.5). An aliquot (1 ml/ml agarose) of 3 M $MgCl_2$ (pH 7.5 with Tris) was percolated into the gel and immediately followed by about 30 ml of MTE. Agarose-immobilized phytochrome was stored at 4 C in MTE containing 0.02% (w/v) NaN_3.

Undegraded phytochrome was conventionally purified to SAR = 0.37 as before (5). Brushite-purified phytochrome was obtained by resuspending the pellet after brushite elution and 200 g/l (33% saturation) ammonium sulfate fractionation (10) in 0.1 M Na-phosphate (pH 7.8). The 33% saturation of ammonium sulfate selectively precipitates undegraded (120,000-dalton monomer weight) phytochrome to the exclusion of degraded (60,000-dalton mol wt) phytochrome as shown first by Correll and Edwards (1) and subsequently confirmed in our laboratory (10, 15). All preparations were stored at −76 C. Undegraded phytochrome quantities were calculated from *A* measurements at 667 nm (11, 12) using extinction coefficients determined by Tobin and Briggs (20).

Crude oat extracts were prepared as before (15), except that clarification was at 27,000*g*. Extracts were used the day of preparation.

Immunoglobulin Preparation. Antidegraded-phytochrome serum was prepared as before (13) except that some rabbits were bled nonterminally 10 and 17 days after boosting. After a 6- to 8-week recovery period, these rabbits were reboosted (0.5 mg phytochrome in Freund's incomplete adjuvant) and similarly re-bled. Antiphytochrome IgGs were adsorbed for 4 to 16 h at 4 C to MTE-washed, agarose-immobilized phytochrome, and then pelleted by a 1,500*g*, 2-min centrifugation. The supernatant serum was decanted and saved for readsorption. The agarose was layered on a G-25 column (10 ml G-25/ml agarose), and washed first with 1 M NaCl, 10 mM MOPS-Tris (pH 7.8), until $A^{1\,cm}_{280}$ declined from about 3.0 to less than 0.03 (usually 20–30 ml), and then with about 2 column volumes of MTE. IgGs were eluted with 1 ml/ml agarose of 3 M $MgCl_2$ adjusted to pH 7.5 with Tris, followed immediately by MTE. The G-25 separates the eluted IgG from $MgCl_2$ in less

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[2] Present address: Section of Biochemistry, Cell and Molecular Biology, Cornell University, Ithaca, New York 14853.

[3] Present address: Botany Department, The University of Georgia, Athens, Georgia 30602.

[4] Abbreviations: PAGE: polyacrylamide gel electrophoresis; SAR: specific absorbance ratio, A_{667}/A_{280} with phytochrome as Pr; IgG: immunoglobulin G; MOPS: morpholinopropane sulfonic acid; MTE: 25 mM MOPS-Tris, 5 mM EDTA (pH 7.5).

than 1 min, thus minimizing possible denaturation. Fractions of 1 ml containing $A_{280}^{1\,cm} > 0.05$ were pooled, concentrated by precipitation with 50% saturation ammonium sulfate, collected by centrifugation, and stored at a concentration of about 1 mg/ml in 0.1 M Na-phosphate (pH 7.8) at −20 C. IgG concentration was determined using $E_{280}^{1\%,1\,cm} = 13.6$ (19).

Antiphytochrome IgGs were both covalently coupled to CNBr-activated agarose (120 mg/mg IgG) and subsequently stored as described above for phytochrome.

Immunochemistry. Ouchterlony double diffusion and immunoelectrophoresis were performed as before (10).

Immunoaffinity Phytochrome Purification. Immobilized antiphytochrome IgGs were mixed with 1 to 2 mg of brushite-purified phytochrome/mg IgG for 15 min at 0 C. Agarose was pelleted by a 2-min, 1,500*g* centrifugation and the supernatant was decanted. Agarose beads containing immunospecifically bound phytochrome were layered on a G-25 column (10 ml G-25/ml agarose), washed first with 1 M NaCl, 10 mM MOPS-Tris (pH 7.8), until $A_{280}^{1\,cm}$ declined from about 2.0 to less than 0.03 (about 100 ml) and then with about 2 column volumes of MTE. Then 1 ml/ml agarose of 3 M $MgCl_2$ adjusted to pH 7.5 with Tris was run into the agarose and immediately followed by MTE. The G-25 separates eluted phytochrome, which moves with the void volume, from $MgCl_2$, which moves with the total volume, in less than 1 min, thus minimizing possible denaturation. Phytochrome-containing fractions were pooled, precipitated with 33% saturated ammonium sulfate to insure that only undegraded phytochrome was collected (1, 10, 15), and centrifuged at 27,000*g* for 15 min. The pellet was resuspended in 0.1 M Na-phosphate, 1 mM EDTA (pH 7.8), clarified by centrifugation, and stored at −76 C.

Gel Electrophoresis. SDS PAGE was performed by a previously described modification (5) of the procedure of Weber and Osborn (21). Purities of phytochrome preparations were estimated by dividing the area of the phytochrome band from the gel absorbance scan by the area of all protein bands.

Isoelectric Focusing. Isoelectric focusing was performed in 4% (w/v) polyacrylamide gels by the procedure of Righetti and Drysdale (17) using pH 3 to 10 ampholine (LKB, 8141). Phytochrome was located after focusing by scanning the gel at 667 nm using a Shimadzu MPS-50L recording spectrophotometer fitted with a custom-built gel transport. Total protein was visualized with Coomassie Blue R using the procedure of Otavsky and Drysdale (8). Gel slices of 0.5 cm were equilibrated with 400 μl of water for 2 days at 4 C and pH was measured (Corning model 10).

Absorption Spectra. Phytochrome *A* spectra were measured in an ice-water-cooled, 1-cm cuvette, using a Shimadzu MPS-50L recording spectrophotometer. Spectra were determined after saturating red (λ_{max} = 667 nm) or far red (λ_{max} = 739 nm) irradiation obtained with Balzer B-40 interference filters.

Gel Exclusion Chromatography. Phytochrome was chromatographed using 0.1 M Na-phosphate (pH 7.8), as before (3), except that Sephadex G-200 was used in place of Bio-Gel P-200.

RESULTS AND DISCUSSION

Antiphytochrome IgGs. IgGs are the only proteins eluted from agarose-immobilized phytochrome as judged by mobility on SDS PAGE (Fig. 1). Purified antiphytochrome IgGs are identical to those present in crude antiphytochrome serum by Ouchterlony double diffusion (Fig. 2, a and b) and immunoelectrophoresis (Fig. 2c). If they had not been identical, only a partial cross-reaction with spurs would have been observed. Some antisera show a minor, contaminating precipitin line by immunoelectrophoretic assay (Fig. 2c, arrow) that is not seen by Ouchterlony double diffusion (Fig. 2, a and b). However, IgGs responsible for this nonphytochrome precipitation are not detected in purified antiphytochrome IgGs but remain in the adsorbed serum (Fig. 2d). Adsorption of antiphytochrome IgGs appears complete since

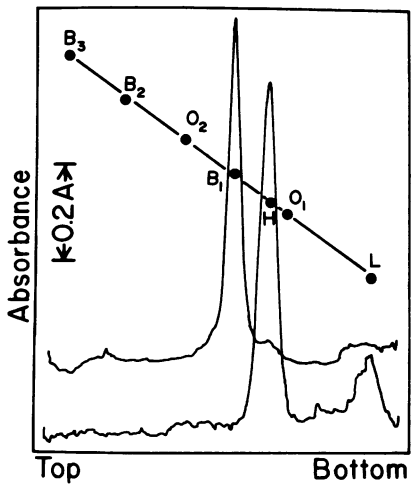

FIG. 1. *A* scans of SDS-polyacrylamide gels after electrophoresis of about 0.1 μl of crude rabbit antiphytochrome serum (about 6 μg protein, upper line) and about 10 μg of affinity-purified antiphytochrome IgGs (lower line). Size standards are run with each experiment. The calibration line presented here is typical of those obtained in other experiments. O_1, O_2: ovalbumin (Sigma A-5503) monomer (45,000 daltons) and dimer, respectively; H, L: bovine IgG (Sigma BG-11) heavy (50,000 daltons) and light (25,000 daltons) chains, respectively; B_1, B_2, B_3: BSA (Sigma A-4503) monomer (66,000 daltons), dimer and trimer, respectively.

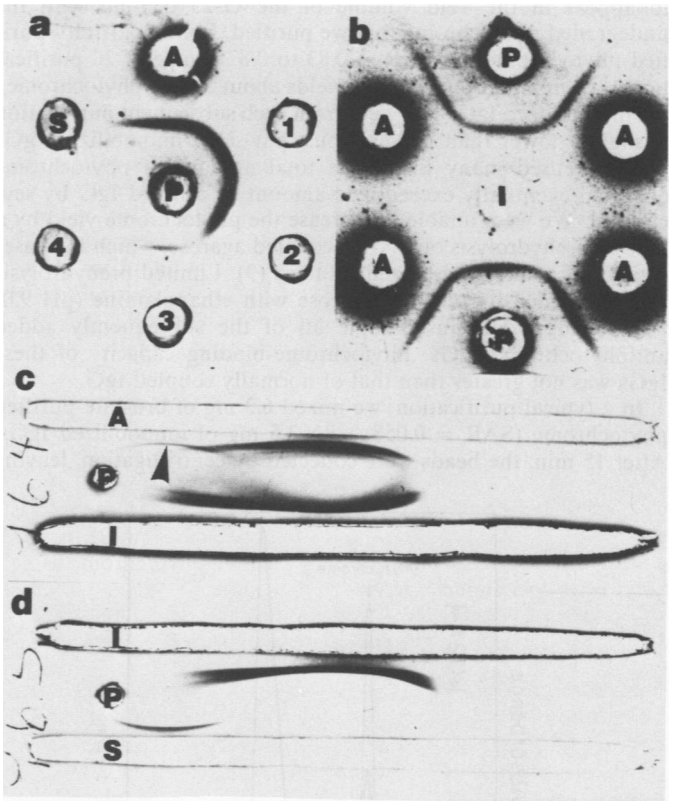

FIG. 2. Ouchterlony double diffusion of: (a) brushite-purified oat phytochrome (P, 0.17 mg/ml) against crude antiphytochrome serum (A), the first four fractions (1–4) from the G-25 column after the void volume during elution of IgG from 1 ml of agarose-immobilized phytochrome, and the same serum after extraction of antiphytochrome IgGs (S); and (b) brushite-purified oat phytochrome against crude antiphytochrome serum and combined, purified antiphytochrome IgGs (I). Immunoelectrophoresis of brushite-purified phytochrome (P, 1.7 mg/ml) against (c) crude antiphytochrome serum and combined purified antiphytochrome IgGs or against (d) combined, purified antiphytochrome IgGs and the original serum after extraction of antiphytochrome IgGs. The upper trough in (c) and the lower in (d) were filled twice to intensify precipitin bands.

the adsorbed serum shows no precipitation of phytochrome by either Ouchterlony double diffusion or immunoelectrophoresis. Quantitative immunoprecipitation, as described by Maurer (7) using [^{3}H]phytochrome (5) to measure pelletable antigen, indicates that at least 50 to 80% of the phytochrome-precipitating activity was recovered from the crude serum (data not shown). The unrecovered fraction might represent immunoglobulins other than IgG that did not precipitate at 50% ammonium sulfate concentration or IgGs that were lost during handling.

IgGs were immobilized on agarose so that only about one-half of the binding capacity of the beads was used. The initial supernatant from the coupling procedure had no detectable phytochrome-precipitating activity by immunoprecipitation or double diffusion assays indicating that coupling of IgGs was virtually complete.

Immunoaffinity-purified Oat Phytochrome. Starting with brushite-purified oat phytochrome (SAR = 0.05, about 11% homogeneous), we obtain undegraded phytochrome (monomer weight = 120,000 daltons) that is greater than 98% homogeneous as judged by SDS PAGE (Fig. 3). The entire purification, from the initial adsorption by agarose-immobilized antiphytochrome IgGs to resuspension of the phytochrome pellet, requires only about 2 h. The rapidity of the procedure as well as the use of 200 g/l ammonium sulfate fractionation eliminates contamination by degraded phytochrome as evidenced by the absence of any band at 60,000 daltons on the SDS gel (Fig. 3). If degraded phytochrome were bound to the immunoaffinity column, it would be expected to appear in the void volume of the G-25 column with the undegraded phytochrome that we purified. Immunoaffinity-purified phytochrome has SAR = 0.83 to 0.87 (range of 20 purifications). Each initial purification yields about 200 μg phytochrome/mg immobilized IgG. The yield from each subsequent purification is slightly lower than the previous, but since immobilized IgGs can be reused many times, the total amount of phytochrome obtained eventually exceeds the amount of coupled IgG by severalfold. We were unable to increase the phytochrome yield by a limited prehydrolysis of CNBr-activated agarose, which increases activity of some immobilized proteins (9). Limited prehydrolysis was performed by titrating agarose with ethanolamine (pH 9.0) until beads would just couple all of the subsequently added antiphytochrome IgGs. Phytochrome-binding capacity of these IgGs was not greater than that of normally coupled IgG.

In a typical purification, we mixed 6.3 mg of brushite-purified phytochrome (SAR = 0.058) with 2.5 mg of immobilized IgGs. After 15 min, the beads were collected by centrifugation, leaving

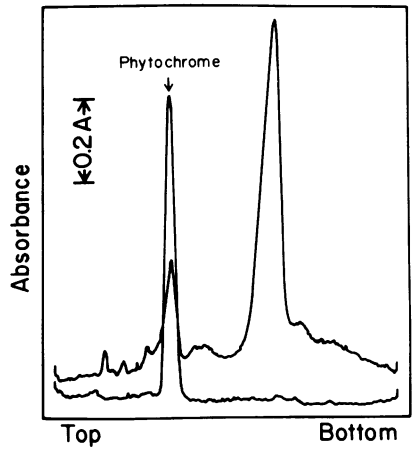

FIG. 3. *A* scans of SDS-polyacrylamide gels after electrophoresis of about 50 μg protein from a brushite-purified oat phytochrome preparation (SAR = 0.05, upper line) and about 30 μg of oat phytochrome immunoaffinity purified from this brushite preparation (SAR = 0.86, lower line). Calibration line was virtually the same as that presented in Figure 1.

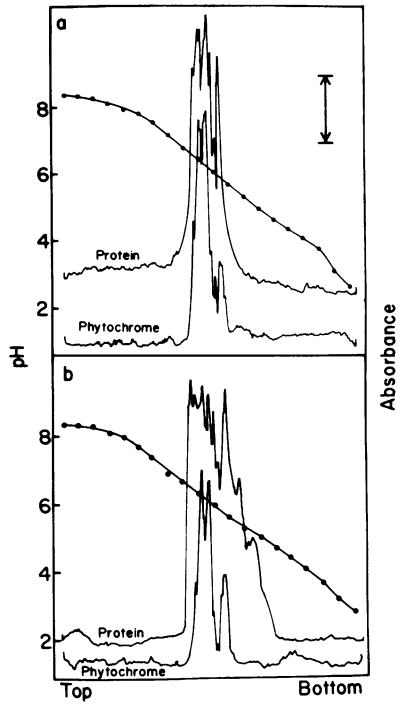

FIG. 4. *A* scans of polyacrylamide gels after isoelectric focusing of (a) 85 μg of immunoaffinity-purified oat phytochrome (SAR = 0.86), and (b) about 170 μg of protein from a conventionally purified oat phytochrome preparation (SAR = 0.37). For phytochrome scans, arrow indicates a $\Delta A_{667} = 0.02$, and for Coomassie blue scans of total protein, a $\Delta A_{505} = 0.2$.

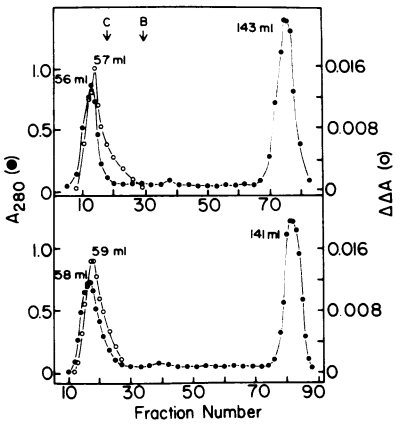

FIG. 5. Elution profiles of immunoaffinity-purified (upper) and conventionally purified (lower) oat phytochrome from a Sephadex G-200 column (1.5 × 85 cm). Applied samples contained about 1 mg phytochrome, 2 mg blue dextran (to mark void volume), and 1 mg tryptophan (to mark total volume) in 2 ml 0.1 M Na-phosphate (pH 7.8). C and B refer to elution peaks of catalase (Sigma C-10) and BSA, respectively.

2.7 mg of phytochrome (SAR = 0.031) in the supernatant. The 30-ml NaCL wash, during which the A_{280} declined from 1.48 to 0.029, contained 2.1 mg of phytochrome (SAR = 0.066). $MgCl_2$ eluted 0.5 mg of phytochrome (SAR = 0.83). At least some of the phytochrome that was not accounted for (about 1 mg) presumably remained adsorbed to the IgGs since the agarose beads turned blue after several purifications, and since each subsequent purification yielded about 20% less phytochrome than the immediately preceding purification.

We have subsequently found that elution with 1 ml/ml agarose of 1 M formic acid after the $MgCl_2$ elution, again followed immediately by MTE, removed from immobilized IgGs phytochrome that would not elute with $MgCl_2$. Agarose was immediately

washed a second time with 1 ml/ml agarose of 1 M formic acid followed immediately by about 50 ml of MTE, and a second time with 1 ml/ml agarose of 3 M $MgCl_2$, pH adjusted to 7.5 with Tris, followed immediately by about 50 ml of MTE containing 0.02% (w/v) NaN_3. No phytochrome is saved from the second formic acid and $MgCl_2$ elutions. Using this revised procedure, from 22 mg of brushite-purified phytochrome (SAR = 0.043) mixed with 20 mg of immobilized IgGs, we obtained 3 mg of phytochrome (SAR = 0.83) by $MgCl_2$ elution and 1.5 mg by formic acid elution in each of four consecutive purifications. Using only 6 mg of brushite-purified phytochrome, we obtained about 1.5 mg by $MgCl_2$ elution and 3 mg by formic acid elution, indicating a difference in binding of phytochrome to IgGs when using different quantities of phytochrome for purification. The SAR of the $MgCl_2$-eluted phytochrome obtained by this procedure is decreased (0.67 to 0.83, range of 15 purifications) compared to that of phytochrome obtained from immobilized IgGs that were exposed to only $MgCl_2$. Phytochrome eluted by formic acid has almost no visible extinction and is insoluble in aqueous buffers at neutral pH. Both of these preparations are as homogeneous as that obtained from beads exposed to only $MgCl_2$ as judged by SDS PAGE (data not shown) indicating that any decrease in SAR represents only spectral denaturation. The formic acid-eluted phytochrome has been used to inject rabbits for antiserum production. The slightly spectrally denatured, $MgCl_2$-eluted phytochrome can be used for physicochemical assays. However, spectral assays probably should utilize phytochrome obtained from IgGs exposed to only $MgCl_2$. In experiments presented here, we used phytochrome obtained from immobilized IgGs that had been exposed to only $MgCl_2$ as described under "Materials and Methods." Phytochrome yield lost by lack of formic acid elution of immobilized IgGs before being used for a subsequent purification was not recovered by later elution with formic acid or sodium thiocyanate.

Comparative isoelectric focusing of immunoaffinity-purified (Fig. 4a) and conventionally purified (Fig. 4b) phytochrome indicates that both preparations have the same range of pI (5.8–6.4) and that isophytochromes in each preparation are distributed in the same relative abundance. All peaks that absorb at 667 nm are photoreversible (data not shown). An *A* scan of protein-stained, focused, immunoaffinity-purified phytochrome shows the same number of peaks, although in different relative heights, indicating that the preparation is very pure and that some isophytochromes have lower visible extinction compared to others. Protein stain for conventionally purified phytochrome shows contaminating proteins that are resolved from phytochrome bands. Since two very different purification procedures yield phytochrome with the same pI range, isophytochromes may be due to different genes coding for the molecule, or may arise from differential posttranslational modifications which include attachment of chromophore(s), phosphorylation (Quail and Pratt, unpublished), and, perhaps, limited protease degradation. Isoproteins, which usually have regulator functions due to different activities, have been purified previously from plants (e.g. 2′,3′-cAMP phosphodiesterase from pea seedling, pIs = 4.3, 4.6, 4.8 [6]).

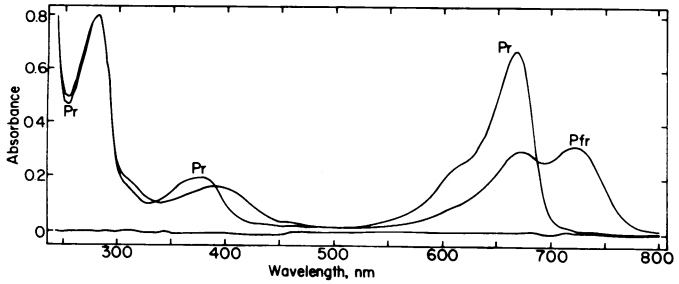

FIG. 6. *A* spectra of immunoaffinity-purified phytochrome (SAR = 0.87, 1.14 mg/ml) after saturating far red (Pr) or red (Pfr) irradiation.

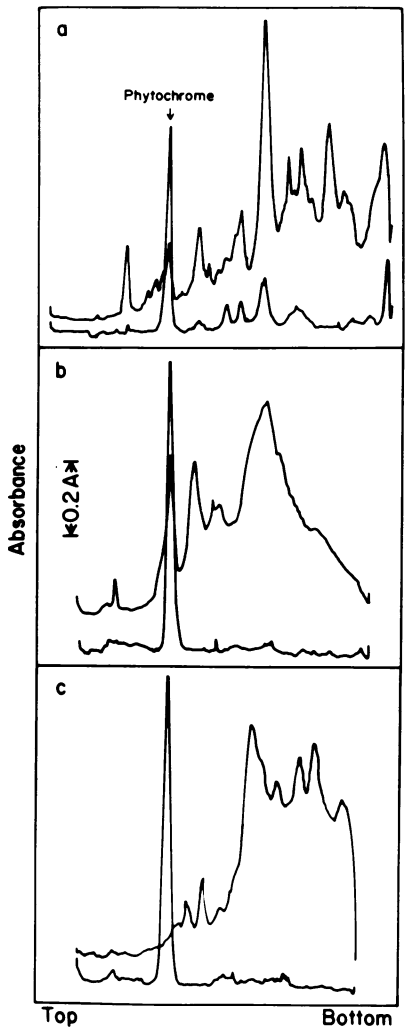

FIG. 7. *A* scans of SDS-polyacrylamide gels after electrophoresis of (a) about 50 μg of brushite-purified (SAR = 0.041, upper line) and about 6 μg of immunoaffinity-purified (SAR = 0.30, lower line) rye phytochrome preparations; (b) about 50 μg of brushite-purified (SAR = 0.043, upper line) and about 5 μg of immunoaffinity-purified (lower line) pea phytochrome preparations; and (c) about 50 μg of crude (upper line) and about 5 μg of immunoaffinity-purified (lower line) oat phytochrome preparations. We could not measure SAR accurately for these crude oat and immunoaffinity-purified pea and oat phytochrome preparations. Calibration lines were virtually the same as that presented in Figure 1.

Gel exclusion chromatography of both conventionally and immunoaffinity-purified phytochrome preparations (Fig. 5) indicates their identity by this assay. Both preparations elute just behind the void volume of G-200 and slightly ahead of catalase. It is evident that exposure to 3 M $MgCl_2$ during immunoaffinity purification does not yield a preparation of phytochrome monomers.

A spectra of immunoaffinity-purified phytochrome (Fig. 6) shows peaks at 280 nm, 382 nm, and 667 nm for Pr and 280 nm, 390 nm, 671 nm, and 724 nm for Pfr, similar to those observed before for conventionally purified oat (10) and rye (12) phytochrome. We have previously observed a similar higher extinction at 724 nm compared to 671 nm (Fig. 6) for degraded oat Pfr (14), as have Rice *et al.* (16) for undegraded rye Pfr, but not for undegraded oat Pfr (14). We cannot explain this apparent discrepancy.

Immunoaffinity Purification of Other Phytochromes. Both rye (Fig. 7a) and pea (Fig. 7b) phytochrome can be partially purified by this technique starting with brushite-purified preparations. Both show apparent monomer weights of 120,000 daltons by SDS

PAGE. Extra peaks may be degradation products of phytochrome, or may be contaminants that are not removed by the 1 M NaCl wash prior to elution.

Purification of oat phytochrome from completely crude extracts demonstrates the potential utility of this technique. This immunoaffinity-purified phytochrome (Fig. 7c) is at least 90% homogeneous, has a monomer weight of 120,000 daltons, and can be obtained in less than 3 h starting with intact oat shoots. Extra peaks are the size of degradation products of phytochrome (data not shown) and therefore may be derived from phytochrome.

CONCLUSION

Phytochrome immunoaffinity purification offers several advantages over other purification procedures. Very high purity phytochrome can be obtained rapidly—in 1 day starting from intact shoots, if desired. Immunoaffinity-purified phytochrome appears identical to conventionally purified phytochrome and is suitable for many physicochemical characterizations that previously were not practical because of the difficulty in obtaining sufficient, homogeneous phytochrome by conventional methods. This immunoaffinity purification technique also has the potential to purify phytochrome from green tissue and, using immobilized antiundegraded phytochrome IgGs, fragments of phytochrome proteolysis.

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