A Simple and Rapid Method for Isolation of 124 kDa Oat Phytochrome

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Z. Naturforsch. **41c**, 988–992 (1986); received June 11, 1986

Avena sativa L., Native Phytochrome, Solubility of Phytochrome

Native (124 kDa) phytochrome has been isolated from etiolated oat seedlings in 25% yield. The procedure includes only one chromatographic step namely a hydroxylapatite column, besides several centrifugation steps. Large scale preparations with 3 kg of fresh tissue are completed within 16 h. The purity of isolated phytochrome is as good as or even better than that of previous preparations as deduced from SDS gelelectrophoresis and from the absorbance ratio $A_{667}/A_{280} = 0.99$.

Introduction

Phytochrome is the main photoreceptor for light-dependent development and differentiation of higher plants [1]. Differential gene expression has been discussed as an important function of phytochrome for a long time [2]. Red light induced changes in the levels of specific m-RNA's [3–6] or the transcription rate of isolated nuclei [7, 8], have been detected in many plant species. The reversibility of such changes by far-red light has been taken as indirect evidence for the participation of phytochrome. The direct influence of purified phytochrome upon *in vitro* transcription in rye nuclei has recently been demonstrated [9]. Whereas transcription was enhanced by undegraded Pfr but not by Pr, the nature of the newly formed m-RNA's and hence the specificity of Pr action in this *in vitro* system has still to be demonstrated.

Experiments along this line need highly purified undegraded phytochrome. The hitherto described procedures for isolation of 124 kDa phytochrome [9–13] are mostly tedious, time consuming or give only moderate yields of pure phytochrome. We describe here a simple and rapid procedure which gives reproducible yields of pure, undegraded phytochrome.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; HA, hydroxyapatite; PEI, polyethyleneimine; PVP, polyvinylpyrrolidone; SAR, specific absorbance ratio = $A^{Pr}_{667}/A^{Pr}_{280}$ for Pr; SDS, sodium dodecylsulfate; Tris, tris(hydroxymethyl)aminomethane; $\Delta(\Delta A)$, difference in absorbance $(A^{Pr}_{667} - A^{Pr}_{730}) - (A^{Pfr}_{667} - A^{Pfr}_{730})$.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/1100–0988 $ 01.30/0

Materials and Methods

The following buffers were used: **A** contains 50% ethylene glycol and 100 mM Tris-HCl, 140 mM $(NH)_2SO_4$, 10 mM Na_4 EDTA, 20 mM sodium bisulfite, and 4 mM PMSF, final pH 8.3 [10]. **B** contains 25% ethylene glycol and 50 mM Tris-HCl, 5 mM Na_4 EDTA, 14 mM 2-mercaptoethanol, and 2 mM PMSF, final pH 7.8 [10]. **C** equals buffer **B** without ethylene glycol and without PMSF [10]. **D** is potassium phosphate, pH 7.8, containing 5 mM EDTA and 14 mM 2-mercaptoethanol; the concentration of potassium phosphate is as follows: $D_1 = 5$ mM, $D_2 = 10$ mM, $D_3 = 100$ mM, $D_4 = 200$ mM.

Phytochrome (124 kDa) was isolated from 3.5 days old etiolated oat seedlings (*Avena sativa* L. c.v. Pirol, Baywa, Munich). Batches of 3 to 4 kg of seedlings were routinely used. The initial steps of our procedure are identical with the method described by Vierstra and Quail [10]. These steps include red irradiation of seedlings immediately before homogenization, extraction with buffer A, precipitation of nucleic acids, pectins and acid proteins with polyethyleneimine, precipitation of phytochrome with ammonium sulfate. We found it necessary to control the pH during or after addition of ammonium sulfate and eventually adjust it to 7.8 with Tris-HCl. The pellet was resuspended in buffer B (4.5 ml per phytochrome unit) and the phytochrome containing supernatant loaded upon a hydroxylapatite (HA) column (size 5 × 8 cm) which had been equilibrated with buffer B containing 70 mM ammonium sulfate [10].

We used commercial HA (Calbiochem, 150–175 ml per batch); we did not find differences between the qualities "fast flow" [13] or "high resolution" for phytochrome purification. After applica-

tion of phytochrome, the HA column was washed with 2–3 column volumes of buffer C. Phytochrome was eluted with a linear gradient made from buffers D_1 and D_4 (5 and 200 mM phosphate, 200 ml each) in a sharp peak. The HA column was re-used after extensive washing with 300 mM potassium phosphate buffer, pH 7.8 (2–3 column volumes) and subsequently with 2 M KCl (1000 ml). All ions have then carefully to be removed from the HA column by excessive washing with distilled water (30–40 l). The column can be re-used 4–5 times after this treatment.

All phytochrome containing fractions ($A_{667}>0.08$) were combined. Phytochrome was precipitated with ammonium sulfate at 42% saturation. The pellet was washed with buffer D_2 (10 mM phosphate, 0.65 ml per phytochrome unit) and then with buffer D_3 (100 mM phosphate, 0.85 ml per phytochrome unit).

The washed pellet was then dissolved in buffer D_2 (10 mM phosphate, 0.9 ml per phytochrome unit). The final purification was achieved by precipitation of phytochrome with polyvinylpyrrolidone (PVP-40, Sigma 1.35 mg per phytochrome unit). The centrifuge tube was carefully rinsed with distilled water (0.2–0.3 ml per phytochrome unit) in order to remove the last traces of PVP. The washed phytochrome pellet was dissolved in buffer D_2 (10 mM phosphate, 1 ml per phytochrome unit). All washing and precipitation steps involved stirring at 4 °C for 20 min and centrifugation at $46.000 \times g$ for 20 min.

The SDS gel electrophoresis is carried out according to Laemmli [14]. Spectra were recorded with a spectrometer model 320 (Perkin Elmer, Überlingen) after saturating irradiation at either 660 or 730 nm [15]. A phytochrome unit is defined as $\Delta(\Delta A)=1$ per ml and cm after saturating irradiation at 660 or 730 nm, respectively.

Results and Discussion

The isolation of 124 kDa phytochrome has been considerably improved by the present method. The procedure which is based upon experience from about forty preparations is summarized in Table I. Whereas the first 2 steps (PEI and ammonium sulfate precipitation) are identical with the procedure of Vierstra and Quail [10], elution from the hydroxylapatitecolumn is achieved here with a linear phosphate gradient. This yields relatively pure phytochrome already at this step. We find the gradient elution superior to the step elution applied by Vierstra and Quail [10]. Datta and Roux [13] also use a gradient at this step which is however complicated (50 mM Tris to 100 mM phosphate). According to our experience, results are more reproducible with the phosphate gradient used in our procedure.

The main improvement of the present procedure over previous methods is the treatment of phytochrome after elution from hydroxylapatite. After precipitation with amonium sulfate at 42% saturation, the precipitate is washed with a defined, small volume of 10 mM phosphate buffer. Phytochrome is not solubilized by this procedure because the concentration of ammonium sulfate is still too high (about 2% saturation). We checked the solubility of phytochrome and found that it is scarcely soluble in 2% saturated ammonium sulfate (Table II). The next washing of the precipitate occurred with 100 mM phosphate in which phytochrome is only slightly soluble (Table II). Both washing steps remove contaminants as demonstrated in Fig. 1. Whereas the supernatant of washing step 1 contains many bands (lane A in Fig. 1), the supernatant of washing step 2 (lane B in Fig. 1) mainly contains a 60 kDa contaminant which is no phytochrome fragment besides

Table I. Purification and yield of phytochrome from 3 kg of fresh oat tissue.

Step	Volume [ml]	A^{Pr}_{667}	Phytochrome units	SAR	Overall yield [%]	Average time elapsed [h]
PEI supernatant	3750	0.02	75	n.d.[a]	100	2
Ammonium sulfate I	330	0.16	52.5	n.d.[a]	70	5
Hydroxylapatite column	225	0.16	36	0.1 –0.15	49	12
Ammonium sulfate II after washing with phosphate	23	0.96	22	0.65–0.87	30	14.5
PVP precipitate	22	0.85	18.5	0.90–0.99	25	16

[a] Not determined because of high and variable background absorption at 280 nm.

Table II. Solubility of 124 kDa oat phytochrome. The values are given as phytochrome concentration in the supernatant at 46.000 g for 20 min in the respective solvent after centrifugation.

Solvent	Phytochrome concentr. [units per ml]	
	Pr-form	Pfr-form
D_2 (10 mM phosphate) containing ammonium sulfate at 2% saturation	0.14	0.09
D at 25 mM	0.26	0.50
D at 50 mM	0.16	0.35
D at 100 mM	0.10	0.14

some 124 kDa phytochrome. Two possible contaminants should be mentioned here: partially degraded phytochrome (114/118 kDa or smaller) is removed by these washings as well as the protease which normally seems to be strongly associated with phytochrome. The presence of this protease was proved by incubation of one unit of purified Pr with the supernatant obtained from 0.05 units of phytochrome: incubation with the supernatant from the first washing step for 54 h at 20 °C results in degradation of 2/3 of 124 kDa Pr into 114/118 kDa fragments. The supernatant of the second washing step yields still traces of the 114/118 kDa fragments under these conditions. Interestingly, purified Pfr is not degraded under the same conditions by incubation with the supernatant from either the first or the second washing step. The stability of Pfr against the protease is in accordance with earlier reports [10–12]. The washing procedures remove the protease completely: the pure product obtained after the washings has been incubated in the Pr form for 54 h at 20 °C or for 2 h at even 36 °C without production of any 114/118 kDa phytochrome or smaller fragments. It is also otherwise stable (see below).

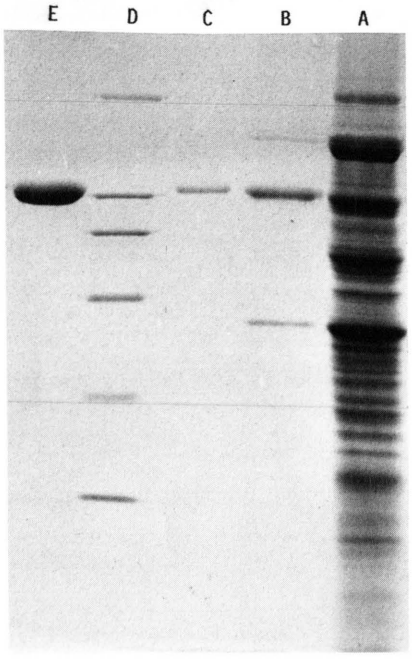

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of aliquots of phytochrome-containing fractions of various stages of purification by the present procedure. Gradient gels containing 7.5–15% acrylamide were prepared according to Laemmli [14] and stained with Coomassie Blue R. lane A, supernatant of first washing with 10 mM phosphate buffer; lane B, supernatant of washing with 100 mM phosphate buffer; lane C and E, phytochrome after final purification with PVP; lane D, marker proteins at 205, 116, 97.4, 66, 45 and 29 kDa.

The final purification step consists of precipitation with polyvinylpyrrolidone (PVP). This treatment was originally planned for removal at an early step of phenolic compounds which eventually modify the phytochrome protein [11]. To our surprise, phytochrome itself is precipitated with PVP. We therefore included this precipitation as the last step of the purification procedure. Removal of phenolics at an early step is certainly essential for yield and quality of phytochrome. According to our experience, this is essentially achieved by the hydroxylapatite column.

The purity of the final product is higher than ever reported in the literature. This can be deduced from the absorption spectrum (Fig. 2). The SAR value of our purest preparations is 0.99, *i.e.* the absorption at 667 nm is about as high as at 280 nm. SAR values previously reported for "pure" phytochrome are about 10% lower (see Table III). Also the absorption at 320 nm is very low in our product; high absorption values at 320 nm point to covalent modification from reactions with oxidized phenolic species [11]. According to this criterion, the product of the present preparation is either not modified in this way or, if so, then considerably less than in previous preparations. Since our procedure avoids the Affi-Gel Blue chromatography [10, 13], the product was never treated with FMN. This also seems to be essential because residual FMN which binds to phytochrome can exert energy transfer to the chromoprotein [16] and in this way eventually cause photo-

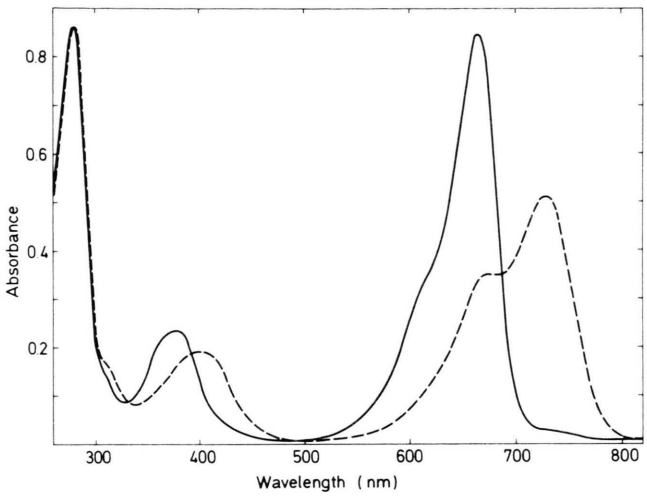

Fig. 2. Absorption spectrum of 124 kDa phytochrome ($A_{667}/A_{280} = 0.99$) in 10 mM phosphate buffer, pH 7.8, containing 5 mM EDTA and 14 mM 2-mercaptoethanol. Absorption spectra were determined at 4 °C after saturating far-red (A) and red (B) irradiation.

Table III. Comparison of purification procedures.

Number of centrifugation steps	Chromatography steps	SAR	Yield [%]	Reference
9	1	0.99	25	this paper
9	3	0.879	17[a]	[11]
8	3	0.90–0.94	13	[10]
7	3	0.80–0.92	10[b]	[13]

[a] Another 3% yield with lower SAR (0.450) was obtained.
[b] Another 5% yield with lower SAR (>0.50) was obtained.

oxidation of phytochrome. We found that our pure phytochrome preparation could be handled at least for several hours at room temperature in bright white light without any loss of spectral absorption, of photoreversibility and without any degradation.

The advantage of our procedure is the use of only one column chromatographic step whereas other procedures include several such steps (see Table III) which are time-consuming. Especially the size exlusion column, which is used by all other authors, requires time and also yields only diluted phytochrome solutions. It is well known that denaturation of proteins is favored by high dilution. The high phytochrome yield of our procedure is certainly inter alia due to the fact that phytochrome is treated only in a precipitate or concentrated solution after the hydroxylapatite step.

The present method does not only give better yields of highly pure undegraded phytochrome but is also much faster than previous procedures [9–11]. It is even faster, especially at large scale preparations, than a previously described rapid procedure [13]. This can be seen by comparison of the initial steps which are essentially identical in the present procedure and in [13]. For PEI precipitation, ammonium sulfate precipitation and HA column chromatography, Datta and Roux [13] estimate 5.5 h when starting with 1 kg of fresh oat tissue. We calculate 12 h for the same steps when starting with 3 kg of fresh oat tissue. Whereas additional 8.5 h were required for further purification steps in [13] we only need additional 4 h for final purification although we treat larger quantities of phytochrome.

The method is mainly based on different solubility of 124 kDa phytochrome and impurities, respectively, in buffers of higher ionic strength. According to our experience pure phytochrome cannot be obtained by application of this principle alone, *i.e.* if the HA column chromatography is omitted. The purification has been worked out for phytochrome from etiolated oat seedlings. It will be interesting to see whether the method is applicable to phytochrome from other sources as well. This work is in progress at present.

Acknowledgements

We thank Miss Marlene Binder for excellent technical help. The work was supported by the Deutsche Forschungsgemeinschaft Bonn-Bad Godesberg.

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