MASTERPARTIAL PURIFICATION OF PHOTOREACTIVATING ENZYME FROM *EUGLENA GRACILIS*

Gary D. Small

Department of Biochemistry, University of South Dakota

Vermillion, South Dakota 57069

SUMMARY: Photoreactivating enzyme has been purified over 400-fold from green cells of *Euglena gracilis*. At this stage in the purification, the nuclease activity of the preparation is reduced to a very low level, permitting the accurate use of the transformation assay for photoreactivating enzyme activity.

INTRODUCTION:

Photoreactivation of ultraviolet light-induced damage has been observed in most organisms lower on the phylogenetic scale than placental mammals (1,2). In many cases it has been possible to show the presence of photoreactivating enzyme activity *in vitro*. The bleaching of *Euglena gracilis* by low doses of ultraviolet light is reversed by exposure of the irradiated cells to visible light (3). The action spectrum for this photoreactivation (3) and the effect of temperature (4) suggest, but do not prove, the involvement of a photoreactivating enzyme.

Higher doses of ultraviolet light kill the cells. This lethal effect of ultraviolet light is also partially reversed by exposing the irradiated cells to visible light. A previous attempt to demonstrate photoreactivating enzyme activity *in vitro* was frustrated by a high level of nuclease activity (5). We have partially purified photoreactivating enzyme from green cells of *Euglena gracilis*. At this stage of purification, the preparation appears to have a very low level of nuclease activity.

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MATERIALS AND METHODS:

Transforming DNA was isolated from a streptomycin resistant strain of *Hemophilus influenzae* Rd by a modification of the method of Muria (6). A 6 hr incubation with pronase (Calbiochem) was included (250 µg/ml, final concentration) following the incubation with pancreatic RNase and RNase T₁ followed by a phenol deproteinization step. The concentration of DNA was determined by the method of Burton (7) using calf thymus DNA (Worthington) as standard.

Protein was determined by the method of Lowry *et al.* (8).

Photoreactivating enzyme activity was assayed by a modification of the method of Muhammed (9). The assay mixture contained in a final volume of 0.6 ml, 0.18 µg of irradiated transforming DNA, 30 µmoles of histidine, pH 6.5, 7.5 µmoles of potassium phosphate, pH 6.5, 0.7 µmole 2-mercaptoethanol, and suitably diluted enzyme. The mixture, in a depression of a white spot plate, was incubated for 10 min at 30° in the dark. A 0.1 ml aliquot was removed for the zero time and the reaction started by turning on the light. 0.1 ml aliquots were removed at subsequent times and assayed for the ability to transform competent *Hemophilus influenzae* to streptomycin resistance (10). The photoreactivating light consisted of two 15 watt G.E. BLB tubes (maximum emission at 366 nm) giving an intensity of 6000 ergs/mm²/min as measured by a long wave UV meter (Ultra Violet Products, Inc., San Gabriel, Calif.). Units of activity are as defined by Muhammed (9).

RESULTS AND DISCUSSION:

Purification of photoreactivating enzyme.

Step 1: Crude extract. *Euglena gracilis* var. *bacillaris* (culture

#160, algae collection, Indiana Univ.) was grown in 12 l batches in Euglena broth (Difco) in the light at 26° with shaking until stationary phase was reached. The cells were harvested by centrifugation (8000 X g), washed twice with distilled water and the packed cells suspended in 4 volumes of 0.05 M KP_i - 10^{-3} M EDTA, pH 6.5. The cells were broken by sonication for 90 seconds with a Blackstone ultrasonic generator set at 35 watts. The mixture was centrifuged for 30 min at 20,000 X g and the pellet discarded. The supernatant solution was frozen at -20°, thawed and centrifuged for 30 min at 20,000 X g. The supernatant solution is the crude extract. The freezing and thawing step removes considerable inactive protein and pigments.

Step 2: Streptomycin precipitation and ammonium sulfate fractionation. To the crude extract, 0.1 volume of 10% streptomycin sulfate was added slowly with stirring. After 15 minutes at 0°, the precipitate was removed by centrifugation (15 min at 20,000 X g). To the streptomycin supernatant solution was added 205 g/l of $(NH_4)_2SO_4$ (35% saturation). The precipitate was removed by centrifugation (15 min at 20,000 X g) and discarded. To the resulting supernatant solution, was added 130 g/l of $(NH_4)_2SO_4$ (55% saturation). The precipitate was removed by centrifugation as above and dissolved in 0.05 M KP_i - 10^{-3} M EDTA- 10^{-3} M 2-mercaptoethanol, pH 6.5 (0.1 the volume of the crude extract).

Step 3: First cellulose phosphate column. The enzyme from step 2 was diluted 1 to 6 with cold distilled water and transferred to a 4.6 x 15 cm cellulose phosphate column equilibrated with 0.01 M NaP_i - 10^{-3} M 2-mercaptoethanol, pH 7.0. The column was washed with

200 ml of the above buffer, 300 ml of 0.1 M KCl in the same buffer, and 300 ml of 0.2 M KCl - buffer. The enzyme was eluted with the above buffer containing 0.5 M KCl.

Step 4: The protein from step 3 was concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (560 g/l), the precipitate dissolved in 0.05 M KP_i - 10^{-3} M EDTA- 10^{-3} M 2-mercaptoethanol, pH 6.2, and diluted to give a protein concentration of about 20 mg/ml. The solution was transferred to a 3X 57 cm column of Sephadex G-100 equilibrated with the same buffer that was used to dissolve the protein and elution carried out with the same buffer. The enzyme is eluted after the major protein peak.

Step 5: Second cellulose phosphate column. The active fractions from the Sephadex column were combined and transferred to a 2.2 X 20 cm cellulose phosphate column equilibrated with 0.05 M KP_i - 10^{-3} M EDTA- 10^{-3} M 2-mercaptoethanol, pH 6.5. The column was eluted with a convex salt gradient (11). The mixer contained 500 ml of 0.05 M KP_i - 10^{-3} M EDTA- 10^{-3} M 2-mercaptoethanol -0.1 M KCl, pH 6.5, and the reservoir contained 0.6 M KCl in the same buffer. The enzyme begins to elute when the KCl concentration reaches 0.35 M in the mixer.

Step 6: Second Sephadex G-100 column. The combined active fractions from the preceding step were concentrated to about 8 ml in an Amicon ultrafiltration cell using a UM 2 membrane filter and transferred to a 5 X 57 cm Sephadex G-100 column equilibrated with 0.05 M KP_i - 10^{-3} M EDTA- 10^{-3} M 2-mercaptoethanol, pH 6.2. The elution pattern is shown in Figure 1. Two activity peaks were observed. The relationship of the two peaks is not known. The fractions from

178 ml to 271 ml were pooled and concentrated in the ultrafiltration cell and referred to as PR A. The fractions from 271 to 343 ml were pooled, concentrated, and referred to as PR B.

Table 1 summarizes the purification procedure of the enzyme from cells harvested from 24 l of media.

Properties of the purified enzyme. Figure 2 illustrates the amount of repair of irradiated transforming DNA vs time of incubation at 30° in the light. No repair is observed when the extract and irradiated DNA are incubated in the dark. The degree of repair of DNA by the *Euglena* enzyme appears to be approximately the same as obtained with the yeast enzyme (11). Tests for nuclease activity indicates a very low level under conditions of our assay. There is no significant inactivation of transforming DNA upon incubation in the dark with 20 times the amount of photoreactivating enzyme normally used for the photoreactivation assay. Table 2 shows that only a small amount of ³H-DNA is solubilized upon incubation with 20 times the amount of enzyme normally used in the photoreactivating assay.

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TABLE I

Purification of Photoreactivating Enzyme from *Euglena gracilis*

Fraction	Protein mg/ml	Total Units	Units/mg	Purification
1. Crude	8.1	119,300	10.9	1.0
2. Streptomycin + (NH ₄) ₂ SO ₄	30.0	101,000	28.0	2.5
3. 1st Cellulose-P	0.98	74,500	205	19
4. 1st Sephadex G-100	0.69	36,300	478	44
5. 2nd Cellulose-P	0.066	24,800	1770	163
6. 2nd Sephadex G-100				
PR A	1.15	10,000	1925	177
PR B	0.69	13,128	4760	437

TABLE 2
Acid Solubilization of ^3H -DNA by Purified
Photoreactivating Enzyme

	<u>Enzyme</u>	<u>Substrate</u>	<u>Acid Soluble cpm</u>	<u>Minus no Enzyme</u>
1.	none	native	121	-
2.	none	denatured	147	-
3.	PR A	native	133	12
4.	PR A	denatured	301	194
5.	PR B	native	272	151
6.	PR B	denatured	785	638

The incubation mixture contained in a final volume of 0.3 ml, 15 μmoles histidine, pH 6.5, 5 μmoles , KPi , pH 6.5, 5.4 μg ^3H -DNA containing 43,000 cpm (isolated from *E. coli* grown in the presence of ^3H -thymidine) and either no enzyme, 57.5 μg of PR A or 34.5 μg of PR B. Incubation was for 60 min at 30° . Reaction stopped by addition of 0.1 ml calf thymus DNA (2 mg/ml) plus 0.3 ml 7% HClO_4 . An aliquot of the acid soluble supernatant solution was counted in a Tri-Carb scintillation counter. The values given are the total acid soluble cpm.

LEGENDS TO FIGURES

FIGURE 1. Chromatography of photoreactivating enzyme on Sephadex G-100. o—o Absorbance at 280 nm, x---x enzyme activity.

FIGURE 2. Time course of repair of irradiated transforming DNA. PR A (2.8 μ g) was the enzyme used. o—o light, --- dark.

2 0.7 11002