The Partial Purification and Characterization of Nuclear and Mitochondrial Uracil-DNA Glycosylase Activities from *Zea mays* Seedlings

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ABSTRACT

Uracil-DNA glycosylase activities from etiolated *Zea mays* seedling nuclei and mitochondria were partially purified and characterized. Nuclei and mitochondria were separated using sucrose differential and step gradient centrifugation. Experiments with osmotically shocked organelles indicated that enzyme activity from mitochondria was soluble, whereas nuclear enzyme activity was only partially soluble under the conditions tested. Purification using DEAE-cellulose and Affigel Blue column chromatography yielded distinct elution profiles from both columns for each of the organellar enzyme activities. Final purification was 490- to 850-fold for the nuclear and mitochondrial uracil-DNA glycosylase, respectively. Characterization studies demonstrated significant differences between the nuclear and mitochondrial uracil-DNA glycosylase with respect to $K_m$ temperature, and pH activity optimum; the effect of salts, and substrate preference. Molecular weight as determined by gel filtration was 18,000 for enzymes from both sources. Both were also sensitive to the sulfhydryl group-blocking agent N-ethylmaleimide. A number of uracil analogs were tested for their ability to inhibit nuclear and mitochondrial uracil-DNA glycosylase activities. 5-Azauracil, uracil, 6-amino-uracil, 6-azauracil, 5-aminouracil, and 5-fluorouracil all inhibited both activities to variable degrees.

Uracil, which is not typically a component of DNA, can be introduced into DNA in vivo by the deamination of cytosine (15), or by the incorporation of dUMP in place of dTMP by DNA polymerases (2). While the incorporation of dUMP, which base pairs with dAMP, appears to have little deleterious effect on cell growth (26), the deamination of cytosine yields a potential transition mutation site at its newly created dUMP-dGMP base pair.

Uracil-DNA glycosylase activity, which removes uracil from dUMP-containing DNA whether base paired with guanine or adenine, was first described in *Escherichia coli* (9, 28). The reaction products of this activity were shown to be free uracil and an unincised DNA strand containing an apurinic site (14). This activity has since been reported from a number of prokaryotic (8, 13, 14) and eucaryotic sources (6, 11, 24), including the higher plant tissue, wheat germ (3), and cultured carrot cells (25). Mitochondrial and nuclear uracil-DNA glycosylase activities have been identified in human KB (1) and rat hepatoma H4 (12) cells.

In this paper we make the first report of nuclear and mitochondrial uracil-DNA glycosylase activities in plants.

 MATERIALS AND METHODS

Materials. Materials for DNA preparations were obtained as follows: calf thymus DNA was from Sigma, poly(dA-dT) from Calbiochem-Behring, and the [5-3H]dUTP (1 mCi/ml, 15 Ci/mmol) used in both preparations was purchased from Moravek Biochemicals.

Column chromatography was performed using DEAE-cellulose from Sigma, and Affigel Blue, and Dowex AG1-X8 from Bio-Rad. Immersible CX-10 ultrafiltration units were purchased from Millipore. Triton X-100, Tween-20, Azure C, protein standards, and ferrocyanochrome C were obtained from Sigma. Mira- cloth was purchased from Calbiochem-Behring.

Preparation of Uracil-Containing Substrates. Nick translated-calf thymus DNA was prepared as described by Krokan and Wittwer (11). Thus, all of the radioactivity was found in [5-3H]dUMP moieties in the DNA. The preparation contained approximately 3000 cpm per 6.8 x 10^{-10} mol dNMP, 1.5% of which were dUMP.

The poly(dA-dT/U) substrate was prepared as described by Blaisdell and Warner (3) with the ratio of dUTP:dTTP in the final product 30:70. The preparation contained approximately 2000 cpm per 1.5 x 10^{-12} mol dNMP, 15% of which were dUMP.

Single-stranded DNA was prepared by heating calf thymus DNA in boiling water for 3 min.

Standard Uracil-DNA Glycosylase Assay Conditions. The standard assay mixture included 0.1 mM N-2-Hepes (pH 7.2), 1 mM EDTA (pH 7.5), 0.01 mg BSA, 3 mM DTT, 0.1% Triton X-100, [5-3H]dUMP labeled poly(dA-dT/U) (4000 cpm, 8.3 x 10^{-7} μmol dUMP), and enzyme (0.5–5 μl per assay) to a final volume of 300 μl. This assay mixture was incubated at 37°C for 15 min. All assays were done within the linear range of uracil removal which extended from 0 to approximately 70% of the substrate uracil content. Incubation was terminated by the addition of 0.8 ml of 0.1 mM NH_4^+HCOO^- (pH 4.3), followed by 10 min on ice. The resulting solution was then applied to an AG1-HCOO^-
column of 0.26 ml volume and the eluate and a subsequent 0.5 ml water wash were collected in a scintillation vial to which 10 ml of Aquasol II was added prior to counting in a Beckman LS7000 scintillation counter. Free uracil, a reaction product, passes through the column as a part of the eluate while mono-nucleotides and polynucleotides remain bound. One unit of activity was defined as the amount of enzyme that catalyzed the release of 1 pmol of uracil per min at 37°C.

Cyt c Oxidase Activity Assay. Cyt c oxidase activity, a mitochondrial marker, was measured as described by Brambl (5). The assay, in a total volume of 1.0 ml, contained 0.1 M K-phosphate (pH 5.9), 1% (v/v) Tween 20, and 15 μM ferrocyanochrome c (>95% reduced). Fractions to be assayed were added to the assay mixture and the initial linear change of A550 with time was recorded.

Protein Assays. All protein assays were performed using the Bradford dye-binding technique which tolerated the levels of Triton X-100 often present and was capable of detecting μg quantities of protein (4).

Plant Growth and Organelle Preparation. Seeds of Zea mays A6327, obtained from the Minnesota Seed Crop Improvement Association, were placed in water overnight, then germinated and grown at 30°C in 12 × 24 inch trays containing well-watered vermiculite. Seedlings were grown in the dark until harvested, 6 to 9 d postimbibition.

Harvested material was cut immediately below the site of extrusion of the adventitious roots, weighed and placed in an ice cold buffer, using 1 g of tissue/ml buffer. Nuclei and mitochondria were isolated from etiolated seedlings harvested in STEB buffer consisting of 0.4 M sucrose, 0.05 M Tris-Cl (pH 7.5), 0.1% (w/v) BSA, and 1 mM EDTA (pH 7.5). Once in STEB buffer, the harvested material was chopped exhaustively with sharp scissors and filtered successively through eight layers of cheesecloth and two layers of Miracloth.

Following an initial 50 g, 5 min centrifugation of the cheesecloth-Miracloth filtrate from etiolated plants to remove cellular debris, a 100 g, 10 min centrifugation was performed to pellet the nuclei. This pellet was twice resuspended in STEB plus 0.25% (v/v) Triton X-100 which lysed plastids and mitochondria but not nuclei, and centrifuged at 1000 g for 10 min to pellet plastid and mitochondrial-free fraction I nuclei.

Mitochondria were isolated as previously described by Lonsdale et al. (16). The 100 g supernatant solution from the nuclear preparation was centrifuged at 12,000 g for 10 min and the resulting pellet was resuspended in 5 ml of STEB buffer and centrifuged 1,000 g for 10 min to pellet contaminating nuclei. This supernatant solution was applied to the top of a 20 ml, 0.6 M sucrose-TEB buffer solution, and centrifuged 10,000 g for 20 min, with the resulting mitochondrial pellet described as fraction I mitochondria.

Each of the nuclear and mitochondrial fraction I pellets were either used immediately or quick frozen at −60°C in an acetone-dry ice bath.

Fraction I organelles were examined under a light microscope for contamination with other DNA-containing organelles, and neither nuclei or plastids visibly contaminated mitochondrial preparations. Assays for the mitochondrial marker, Cyt c oxidase activity, as well as azure C staining of nuclei supported the observation of no cross-contamination.

Column Chromatography. Fraction I nuclei and mitochondria were applied first to 5 ml DEAE-cellulose and then Affigel Blue columns, both of which were equilibrated in KEB buffer consisting of 2 mM K-phosphate (pH 6.5), 10 mM 2-hydroxyethylmercapitan, 1 mM EDTA (pH 7.5). Prior to application to the DEAE column, 1% (v/v) Triton X-100 was added to the mitochondrial fractions which were held on ice for 30 min, and subsequently diluted 10-fold with column starting buffer. The nuclear fraction was simply diluted 10-fold with column starting buffer, and stirred on ice 30 min prior to application to the column. A 50 ml 0.002 to 0.1 M K-phosphate (pH 6.5) gradient with a 2.5 ml 0.5 M K-phosphate (pH 6.5), final wash was run through the DEAE column. Fractions were collected and assayed for uracil-DNA glycosylase activity, protein content, and ionic concentration. Peak activity fractions were pooled, diluted to 2 mM KEB buffer and concentrated using a Millipore immersible CX-10 ultrafiltration unit. This concentrate, fraction II, was in turn applied to an Affigel Blue column and a 50 ml 0.002 to 0.8 M K-phosphate (pH 6.5) gradient was run. Again, fractions were collected, assayed for uracil-DNA glycosylase activity, and conductivity was measured. Peak activity fractions were concentrated as described for the DEAE activity-containing fractions. These concentrates, described as fraction III were used in the characterization studies.

RESULTS

Triton Treatment of Whole Organelles. Taking advantage of the fact that mitochondrial membranes are dispersed by Triton X-100, a uracil-DNA glycosylase assay of fraction I mitochondria

![Fig. 1](https://via.placeholder.com/150)

FIG. 1. Triton treatment of mitochondrial fractions. Intact mitochondria were assayed for uracil-DNA glycosylase activity using standard assay conditions except that the assay mix was made 0.5 M sucrose to maintain organelle integrity during the assay, and Triton X-100 levels were varied from 0 to 1% (v/v) in order to determine the effect on enzyme activity of mitochondrial membrane lysis. The addition of up to 1% Triton X-100 to osmotically shocked mitochondrial extracts demonstrated no stimulatory effect on enzyme activity (data not shown).

Table 1. Suborganelle Distribution of Uracil-DNA Glycosylase Activity

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Treatment</th>
<th>Fraction</th>
<th>Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Osmotic shock</td>
<td>1K pellet</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1K supernatant</td>
<td>48</td>
</tr>
<tr>
<td>Reshocked and</td>
<td>Homogenized 1K</td>
<td>2nd 1K pellet</td>
<td>43</td>
</tr>
<tr>
<td>Homogenized 1K</td>
<td></td>
<td>pellet supernatant</td>
<td>8</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Osmotic shock</td>
<td>3K pellet</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90K pellet</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90K supernatant</td>
<td>79</td>
</tr>
</tbody>
</table>
was performed in the presence of increasing amounts of Triton X-100 using standard assay mixtures that had been made 0.5 M sucrose so as to maintain membrane integrity in the absence of added Triton X-100. Assays with mitochondria treated with 0.5% or 1% (v/v) Triton X-100 demonstrated 5-fold increases in activity when compared to non-Triton X-100 treated mitochondria (Fig. 1). Maximal activity was reached at 0.5% (v/v) Triton X-100, inferring total mitochondrial membrane dispersal by this concentration of Triton X-100. This result indicated that the uracil-DNA glycosylase appeared to be sequestered within the organelle tested, being liberated only by membrane dispersal. Nuclei, which are not disrupted by Triton X-100, were not similarly tested.

Suborganellar Location of Activity. Fraction I nuclei were osmotically shocked by diluting them 10-fold in ice cold KEB buffer. After 30 min with constant stirring this solution was centrifuged 1000g for 5 min and the resulting pellet and supernatant were assayed for uracil-DNA glycosylase activity. The activity was essentially equally divided between the two fractions (Table I). The pellet was subsequently rediluted 10-fold in KEB buffer, ground with a homogenizer, recentrifuged as before, and this pellet and supernatant were assayed for uracil-DNA glycosylase activity, with the vast majority of the activity remaining in the osmotically shocked-homogenized pellet. Sonication of the initial 1000g pellet yielded similar solubilization ratios (data not shown). This indicated that at least half of the nuclear activity was soluble with the remainder possibly associated with some type of complex that was most likely membrane-bound.

Fraction I mitochondria were osmotically shocked by a 30-fold dilution in 0.1 M K-phosphate (pH 7.2) 0.02% (w/v) BSA, and placed on ice for 15 min with intermittent stirring. Centrifugation at 3,000g for 10 min pelleted unbroken mitochondria and ghosts. The 3,000g supernatant solution was then centrifuged at 90,000g for 30 min to pellet inner and outer membranes. The final supernatant solution was described as containing matrix or soluble proteins (17). Nearly all of the uracil-DNA glycosylase activity recovered from lysed mitochondria is located in the matrix rather than bound to the membrane (Table I).

**Purification.** The results of the purification of uracil-DNA glycosylase activity from fraction I nuclei and mitochondria are given in Table II, and a comparison of their elution profiles has been made in Figure 2.

Nuclear and mitochondrial uracil-DNA glycosylase activities were purified 490- and 850-fold, respectively. In both cases, DEAE-cellulose chromatography separated more than 97% of the proteins from the uracil DNA glycosylase activity peak with the majority of the proteins remaining bound to the column prior to a 0.5 M salt wash. DEAE-cellulose chromatography was selected for use based on the belief that the organelle preparations were rich with DNA that needed to be removed before assaying for the desired glycosylase activity. In fact, the increase in activity units for the mitochondrial preparation may be partly due to the separation of organelle DNA from uracil-DNA glycosylase moieties.

The major elution peak from DEAE-cellulose chromatography for mitochondrial glycosylase activity was at 0.067 M K-phosphate whereas that of the nuclear activity was slightly higher at 0.08 M. These distinct elution profile peaks were also obtained when phosphocellulose chromatography preceded DEAE-cellulose chromatography (data not shown).

Subsequently, the affinity chromatography matrix, Affigel Blue, which has a demonstrated affinity for glycosylases from other sources (RJ Bensen, HR Warner, unpublished data), yielded strikingly distinct elution profiles for both organelar uracil-DNA glycosylase activities. When 0.1% (v/v) Triton X-100 was added to the gradient buffer the nucleic activity eluted predominantly at 0.4 M. Without Triton X-100 the nuclear activity failed to bind. Mitochondrial uracil-DNA glycosylase activity bound strongly to the Affigel Blue column, eluting at 0.63 M with or without 0.1% (v/v) Triton X-100. Protein levels of individual Affigel Blue column fractions were too low to be accurately determined.

**K_m.** The K_m of the uracil-DNA glycosylase activities was determined under standard assay conditions using increasing concentrations of poly(dA-dT/U) as the substrate. The results, shown in Figure 3, yielded K_m values of 7.4 × 10^{-3} M and 1.2 × 10^{-6} M.
for the nuclear and mitochondrial activities, respectively.

**Molecular Weight Determination.** Nuclear and mitochondrial uracil-DNA glycosylase fraction II preparations, were applied separately to a Sephadex G-100 column (1.4 x 60 cm) equili-

**pH.** A profile demonstrating the effect of the assay pH on uracil-DNA glycosylase activity from the two organelles is given in Figure 5. The mitochondrial activity demonstrated an optimal pH of 7.0. The nuclear activity, whose optimal pH was 7.5, showed the widest tolerance of pH with 50% or greater activity at pH 6.0 and 8.0.
Uracil-DNA glycosylase activities, the results indicated that the nuclear enzyme removed uracil at an equal rate from both substrates, while the mitochondrial activity was slightly more active on single-stranded DNA, removing uracil from double-stranded DNA at a rate only 87% of that observed with single-stranded DNA (data not shown).

Uracil Analogs. A number of analogs to uracil, a reaction product, were tested for their ability to inhibit uracil-DNA glycosylase activity. Those analogs which inhibited either nuclear or mitochondrial activities at 1 mM concentrations are listed in Table III. Analogs which demonstrated no inhibitory capacity included: cytosine, thymine, orotic acid, uridine, 2'-deoxyuridine, dUMP, 6-methyl uracil, 5-hydroxy uracil, 5-hydroxymethyl uracil, 2-thiouracil, and 3-methyluracil.

DISCUSSION

This paper represents the first report of uracil-DNA glycosylase activity located in the nucleus and mitochondrion of plant cells.

We have described the partial purification, characterization, and comparison of uracil-DNA glycosylase activities from two of the three DNA-containing organelles of Zea mays cells, the nuclei and mitochondria. We will describe separately a maize chloroplast uracil-DNA glycosylase activity (RJ Bensen, HR Warner, unpublished data). Based on differences observed during subcellular fractionation, and activity purification and characterization, we have concluded that the maize seedling cell contains at least three distinct uracil-DNA glycosylase activities.

Uracil-DNA glycosylases have been characterized from a number of phylogenetically diverse organisms, and although a number of physical and catalytic characteristics of the activity, such as mol wt, $K_m$, sulfhydryl group blocking agent effects, substrate preference, and activity inhibitors, have been shown to vary to some degree from organism to organism, no evolutionary pattern for the differences has been apparent. Our results support the lack of a discernible evolutionary pattern, as the mitochondrial activity seems no more closely related to procaryotic activities than to the maize nuclear activity.

Of the parameters measured, only two yielded common properties in both organelles. In the first case, using gel filtration chromatography, the mol wt of both activities was determined to be 18,000. This predicted mol wt fell within, but at the lower limit of, the 18K to 30K range of those previously reported, and is very different from the reported difference between a 35K nuclear and a 20K mitochondrial activity from rat hepatoma cells, also determined by gel filtration (12). The common apparent mol wt of both activities is interesting when one considers the fact that, based on known organelle-DNA coding capacities, the likelihood that the mitochondria code for their own uracil-DNA glycosylase activity is remote. Thus, it is probable that both are nuclear coded, in which case the mitochondrial activity must be transported into mitochondria, suggesting it is synthesized as a larger precursor which is subsequently processed to a smaller mature form (7, 22).

The second similarity between both activities was their sensitivity to the sulfhydryl group blocking agent, NEM, demonstrating a greater than 50% loss of activity at 10 mM NEM concentrations. Such sensitivity to NEM has not been observed for any other uracil-DNA glycosylase, from either procaryotic (8, 14) or eucaryotic (3, 11, 24) origin. Although not sensitive to NEM, most uracil-DNA glycosylases, including the Escherichia coli activity, have been found to be sensitive to inhibition in the presence of the hydrophobic sulfhydryl group blocking agent, p-mercuribenzoate. Interestingly, E. coli activity, which is the only uracil-DNA glycosylase whose amino acid content has been determined, contains only one cysteine residue (14). The T4-induced UV endonuclease, which contains a pyrimidine dimer-DNA glycosylase and an AP endonuclease activity, also contains...
only one cysteine, whose sulfhydryl group blocking agent sensitiv-
ity is unknown (21, 27). The sensitivity of uracil-DNA glyco-
sylase activities to NEM or p-mercuribenzoate is intriguing when one considers the mechanistic models for N-glycosidic cleavage, which point to a nucleophilic attack by the enzyme on the 6-
position of the base to be removed (10, 19, 20). Cysteine is an excel-
llent candidate to be such a nucleophile as is clearly dem-
strated by thymidylate synthetase, whose cysteine covalently
binds to the 6-ring position of dUMP as a step in the conversion of
dUMP to dTMP (18).
Upon osmotically lysing the nuclear and mitochondrial orga-
nelle preparations to determine the suborganellar location of
their respective uracil-DNA glycosylase activities, the mitochondri-
al activity was found to be at least 79% solubilized and we
thus concluded it was primarily a free matrix protein. On the other
hand, the nuclear activity was nearly equally distributed be-
tween soluble and insoluble fractions, a result that re-lysis and
fractionation did not significantly alter.
In comparing the effect of pH, ionic strength, MgCl₂, and
EDTA on the two uracil-DNA glycosylase activities, nuclear
activity was unchanged by NaCl up to 10 mm, but at higher
concentrations activity diminished, while mitochondrial activity
was stimulated by 10 to 20 mm NaCl and was more gradually
inhibited by higher concentrations. Similarly, the rat hepatoma
mitochondrial activity was shown to be optimally active at a
higher NaCl concentration (40 mm) than its nuclear counterpar-
t (20 mm) (12).

The $K_a$ values of 7.4 and 12.0 $\times 10^{-7}$ M for the nuclear and
mitochondrial activities, respectively, represented a small but
repeatable difference. Previously reported uracil-DNA glycosyl-
ase $K_a$ values have been the same or smaller, ranging from $7 \times
10^{-7}$ M (12, 24) to 1.1 $\times 10^{-9}$ M (8).
While procaroytic uracil-DNA glycosylases prefer single-
stranded DNA (8, 13, 14), eucaryotic activities have been shown
to prefer single-stranded DNA in one case (11) and double-
stranded DNA in two others (3, 24). For the maize uracil-DNA
glycosylases, the nuclear activity removed uracil at an equal rate
from either substrate, while the mitochondrial activity was
slightly more active on single-stranded DNA (1.2:1).

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