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Deoxyribonuclease Activity from Acetone Powder Preparation of *Chlamydomonas reinhardii*

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Deoxyribonuclease activity has been extracted from acetone powder preparation of *Chlamydomonas reinhardii* protoplasts. The enzyme preparation does not show an absolute requirement for divalent cations, although addition of Mg^{2+} and Ca^{2+} results in 1.5- and 3-fold stimulation, respectively. The enzyme preparation has two *p*H optima at 7.0 and 10.0, and prefers double stranded DNA over denatured DNA as a substrate. The nuclease activity remains stable for about three weeks at −16°C, however, repeated freezing and thawing results in a 50% loss of the enzyme activity in six days.

Key Words: Polycations, *Chlamydomonas reinhardii*, Acetone powder, DNase, Isolation and characterization.

Introduction

Polycations such as spermine, spermidine, poly-L-ornithine, poly-L-lysine etc. stimulate the uptake of exogenous DNA by *Chlamydomonas* and other plant protoplasts (Upadhyaya & Hasnain 1980). Recently, attempts have been made to elucidate the mechanism of such a stimulation (Hasnain & Upadhyaya 1980, Hasnain et al. 1980). Some initial observations on the interaction of polycations with DNase, obtained from commercial sources, indicated an inhibition of the enzyme activity. In order to extend these findings to in vivo conditions a DNase activity was isolated from *Chlamydomonas reinhardii*. Although a considerable amount of information exists on nucleases isolated from bacterial and animal systems, very little is known about plant (Johnson & Laskowski 1968, Wilson 1975, Jenns & Bryant 1978 and Wani & Hadi 1978) and algal nucleases (Schonherr et al. 1970, Burton et al. 1977). Most of the results on plant nucleases are based on assays which are essentially extension of those worked out for bacterial and animal nucleases. In this communications, we report a

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preliminary characterization of nuclease activity from acetone powder extracts of *Chlamydomonas* protoplasts, in terms of *p*H dependence, divalent cation requirements, substrate specificity etc.

Materials and Methods

Chlamydomonas reinhardii CW 3+C and *Escherichia coli* HF 958 leu⁻thy⁻ were obtained from Dr P M Gresshoff, Australian National University and Prof. H K Das, Jawaharlal Nehru University; ^{3}H-methyl thymidine (13 Ci/m mol) was purchased from Bhabha Atomic Research Centre, Bombay; and most of the biochemicals were obtained from Sigma Chemical Co., USA.

Preparation of Enzyme Extract: Chlamydomonas reinhardii CW 3+C was grown as described (Gresshoff 1976). All subsequent operations were carried out at 0–4°C. Cells in mid-log-phase were harvested by centrifugation, washed once with the culture medium, suspended in cold acetone (−16°C) and subsequently sonicated for 30 seconds in an MSE ultrasonic disintegrator (8.0 mm probe, 12μ amplitude).

The dark green suspension was washed with excess of cold acetone by filtration on a Whatman 40 filter paper to remove the pigments etc., and dried in a vacuum dessicator to eliminate the traces of acetone. About 6 litres of log phase culture yielded 1 g of dry acetone powder by this method.

Acetone powder was suspended in (1:25, w/v) 0.15M NaCl containing 10 mM mercaptoethanol and stirred for 30 min in cold room. The suspension was spun at 5000 × g in Sorvall RC-5 centrifuge to pellet the insoluble material. The clear supernatant was adjusted to 40% $(NH_4)_2SO_4$ saturation and stirred mechanically for 30 min. The precipitate was discarded after being separated by centrifugation at 10,000 × g for 15 min. The second supernatant was then adjusted to 50% $(NH_4)_2$ SO_4 saturation and recentrifuged at 10,000 × g to collect the protein pellet. This protein pellet was redissolved in 2–3 volumes of saline mercaptoethanol and eluted with the same solution through a pre-equilibrated 20 × 1.5 cm Sephadex G-25 column. The fractions containing enzyme activity, migrating as a single visible band, were collected manually and stored frozen.

Assay for DNase activity: ^{3}H-DNA as well as cold DNA solutions were used as substrates for the enzyme. ^{3}H-DNA was isolated from *E. coli* HF 958 leu⁻thy⁻, using an earlier method (Uchimiya & Murashige 1977) with the following modifications. The final concentrations of lysozyme, pronase and sarkosyl were 100 μg, 1 mg and 1 mg per ml respectively. For deproteinization, Sevag reagent was replaced by chloroform-isoamyl alcohol (7:3). One more deproteinization step was added after RNase digestion. This was followed by dialysis of the purified DNA against SSC (0.15 M NaCl and 0.015 M trisodium citrate). The medium (Uchimiya & Murashige 1977) was supplemented with thymine (10μg/ml) and L-leucine (20 μg/ml) as and when required. The yield of the purified ^{3}H-DNA (A260/280 = 1.6, specific activity 2100 dpm/μg DNA) was about 1.5 mg as compared to 600 μg obtained by the original method (Uchimiya & Murashige 1977). Unlabelled DNA was isolated and purified by a similar method except that thymine (10 μg/ml) was substituted for ^{3}H-methyl thymidine. DNase activity was monitored by estimating TCA soluble materials released from DNA as a result of nuclease digestion. The assay mixture contained ^{3}H-labelled *E. coli* DNA as substrate, 0.1 M

Tris-HCl buffer, *p*H 7.0, and 6mM $CaCl_2$. Other details are given in the legends for the figures. The reaction carried out at 25°C in a 3 ml system, was started by addition of the enzyme fraction and normally terminated after 10 min. with 7% TCA (w/v). In control experiments, TCA was added to the reaction mixture before the enzyme. The acid soluble material was determined by reading the increase in absorbance at 260 nm against appropriate blanks. In assays employing ^{3}H-DNA as substrate, the assay mixture, after the termination of the reaction, was filtered through Maxflow membrane filters (45 μ). The Filters were dried and then counted in a toluene-based scintillant (0.4% PPO and 0.05% POPOP) in a Packard Tricarb Scintillation Spectrometer. Acid soluble radioactivity was computed by substracting the activity retained on the filters from the total activity (in controls). Denatured DNA was prepared by the conventional method of heating the DNA solution at 100°C for 8 min, followed by rapid cooling to 0°C. Protein was estimated by the Warburg Christian equation (Herbert et al. 1971).

Results and Discussion

The acetone extraction was preferred over aqueous methods to ensure the removal of tannins and quinones from the homogenate before they could interact with proteins. Acetone also removes photosynthetic pigment complexes (Rhodes 1977), which are known to interfere in enzyme purification from algae. The $(NH_4)_2$ SO_4 cut between 40–50% led to 3.5 fold enrichment of the enzyme. The enzyme preparation was passed through Sephadex G-25 column to remove $(NH_4)_2$ SO_4 and residual phenolics. The temporal profile of the enzyme activity is shown in figure 1. Since the reaction was found to approach a plateau after 10 min, the reaction time was fixed at 10 min. for later experiments.

The *p*H profile of the DNase activity is shown in figure 2a. The enzyme preparation showed bimodal *p*H optima, one at *p*H 7.0 and another at *p*H 10. A minor activity was also present at *p*H 4.0. The enzyme preparation was active even in the absence of Mg^{2+} and Ca^{2+} ions (figure 2b) indicating that it did not have an absolute requirement for divalent cations. However, the addition of Mg^{2+} and Ca^{2+} to the reaction mixture led to 1.5- and 3-fold stimulation, respectively, in the nuclease activity. Optimal activity (inset figure 2b) was recorded in presence of 6 mM Ca^{2+}. The preparation was more active with double-stranded DNA, although it was significantly active with single-stranded DNA also (figure 2c). The use of a mixture of double and single-stranded DNA in the ratio of 1:1 (without changing the total amount of DNA) resulted in a decrease in the overall activity.

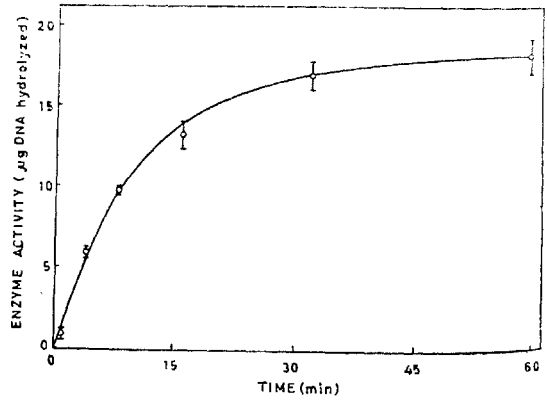

Figure 1 Temporal profile of DNase isolated from *Chlamydomonas*. The activity of DNase preparation (150–200 μg protein) was monitored using ^{3}H-DNA (8 μg/ ml) of *E. coli* as substrate

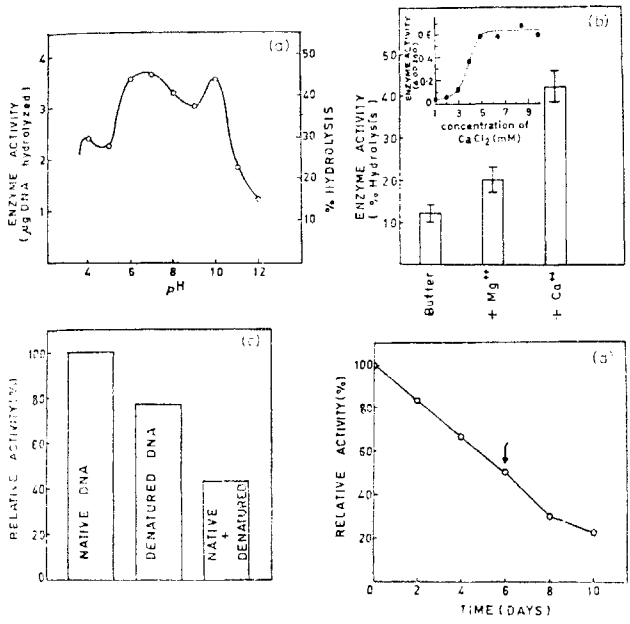

Figure 2 The DNase activity was assayed using 6–2 µg/ml of *E. coli* ^{3}H-DNA as substrate and 150–200 µg protein of the enzyme preparation; *(a)* *p*H profile of the DNase. The enzyme was assayed in 0.1M each of acetate, Tris-HCl, glycine-NaOH, and KCl-NaOH buffers in the *p*H range of 4–5, 6–8, 9–10 and 11–12 respectively; *(b)* Influence of divalent cations on the DNase activity. The activity was monitored for 10 min in the presence of 6mM of $MgCl_2$ and $CaCl_2$ in the assay mixture. Inset: The effect of Ca^{2+} concentration on the enzyme activity was studied by varying the concentrations of $CaCl_2$ in the reaction mixture; *(c)* Substrate preference of the enzyme. The reaction was carried out using native DNA, denatured DNA, and 1:1 mixture of native and denatured DNA as substrate; *(d)* Effect of repeated freezing and thawing on activity of the enzyme assayed after storing it at 16°C

The nuclease remains stable for at least three weeks at −16°C, however, frequent freezing and thawing caused a rapid loss in activity. About 50% of the activity was lost in about 6 days (figure 2d) under these conditions.

The increase in DNA concentration up to 200 µg/ml in the reaction mixture led to a proportionate increase in the rate of hydrolysis of the substrate (figure 3). Substrate concentrations below 50 µg/ml showed a very low enzyme activity. Effect of increasing enzyme concentration on DNase activity gave identical results (figure 4). The kinetics of DNA degradation in relation to enzyme and substrate concentrations can be explained in a number of ways. The absence of activity at low concentration of the enzyme could be explained if one assumes the requirement of an activating cofactor for the enzyme—as the protein

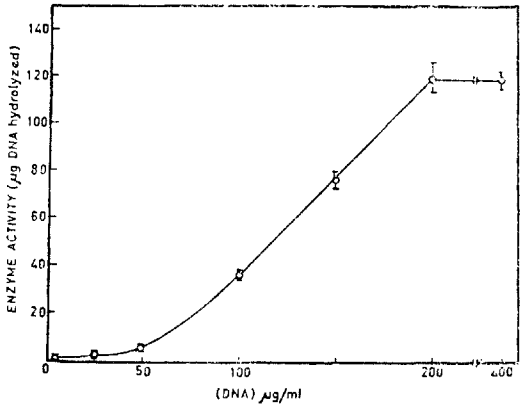

Figure 3 Effect of increasing substrate concentration on DNase activity

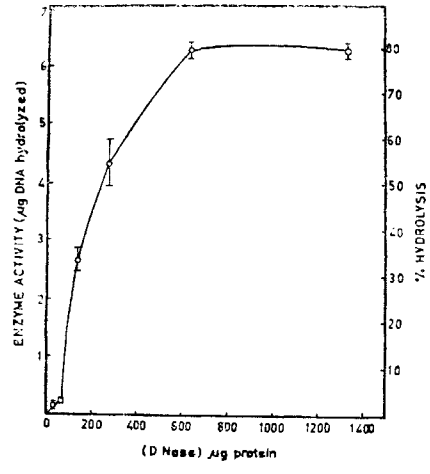

Figure 4 Effect of increasing enzyme concentration on DNase activity

concentration is increased, the quantity of this co-factor reaches the critical threshold limit thereby allowing the reaction to proceed. It can also possibly mean an allosteric activation of the enzyme and the sigmoid dependence of V upon the concentration of the substrate is an indication of such an effect. Another instance in which sigmoidal curves can occur is when enzyme solution contains an impurity that combines irreversibly with the subtrate (Westley 1969). In this case no velocity will be observed until sufficient substrate has been added for it to exceed the amount of the irreversible inhibitor present. Thus, it is reasonable to suggest, that the apparent substrate induction is due to some impurities present in the enzyme preparation.

The multiple *p*H optima of the nuclease activity and its stimulation by both Ca^{2+} and Mg^{2+} are indicative of the presence of at least two enzymes in the preparation. Substrate specificity also points in this direction. Possibly there are two DNases: One having a *p*H optimum of 7.0, requiring Mg^{2+} and specific for native DNA, and the other, with a *p*H optimum of 10.0, requiring Ca^{2+} and specific for single-stranded DNA (SS-DNA).

The existence of an "Acid DNase" (*p*H) optimum 4.6 with DNase II like properties) and an "Alkaline DNase" (*p*H optimum of 9.6 with preference for heat denatured DNA) in *Euglena* lends support to such a postulation (Carell et al. 1970). While the former is known to be inhibited by higher concentration of Ca^{2+}, Mg^{2+} and Mn^{2+}, the latter requires Ca^{2+} (3×10^{-2}M) for optimal activity. In our preparation, however, Ca^{2+} was not found to have any inhibitory effect on the former activity. The minor activity at *p*H 4.0 could also be comparable to nuclease A of *Aspergillus niger* (Saruno et al. 1979). SS-DNA-specific DNase activities have been reported from plants (Hanson & Fairley 1969 and Kroeker et al. 1975) and even a Ca^{2+}-stimulated SS-DNase is known to exist in peas (Wani & Hadi 1979).

The nomenclature of plant nucleases is far too complicated because nuclease preparations have rarely been shown to be DNA-specific. Mung bean nuclease being a non-specific moiety, hydrolyses single stranded derivatives of ribose, deoxyribose and arabinose, preferentially (Mikulski & Laskowski 1970). Similar findings have been reported from *Avena* leaf tissue. However, in this case Zn^{2+} was found to inhibit RNase activity without affecting the properties of DNase (Wyen et al. 1971). The location of the alkaline DNase within the cell is still a matter of speculation. This enzyme, because of its increased activity during the light-induced chloroplast development in dark adapted *Euglena* cells, can in all probability be assigned to chloroplast (Carell et al. 1970). Such a high *p*H optimum-nuclease has also been reported from chloroplasts (Hadziyev et al. 1969).

Recently a restriction endonuclease system has been implicated in maternal inheritance (Burton et al. 1977). The work is in progress in isolating, purifying and characterizing the different nuclease activities reported in our preparation.

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