Deoxyribonuclease activities of wheat seedlings

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A rapid and sensitive method for the assay of DNases is described. This method has revealed that homogenates of wheat seedlings contain a DNase which is activated by Mg^{2+}, and another which is activated by EDTA. Thus EDTA alone will not protect DNA from cleavage during its isolation from wheat seedlings. Both activities have their pH optima at pH 7.0. Trypsin did not destroy the DNase activities of homogenates, but diethyl pyrocarbonate was an effective inhibitor.

Triticum aestivum *DNase assay* *DNA isolation* *EDTA-activated DNase*

1. INTRODUCTION

The standard procedure for obtaining high molecular weight chloroplast DNA from plants involves the isolation of intact chloroplasts in aqueous suspensions, followed by lysis of the chloroplasts, using EDTA to inactivate deoxyribonucleases (DNases) [1]. This method gives high yields from a number of dicotyledonous plants, such as tobacco, spinach and peas, but is, at best, unreliable when applied to certain cereals, such as wheat. This may be due in part to the difficulty of isolating intact chloroplasts from the tougher leaves of cereals, but it may also result from the presence of higher levels of endogenous DNase activity in the tissues of cereals. Using the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) to stain chloroplast DNA, Sellden and Leech [2] have shown that the levels of endogenous DNases are higher in wheat than in spinach or tobacco. A non-aqueous method for the isolation of wheat chloroplast DNA has been described [3], and it is likely that the success of this method results from an efficient inactivation of endogenous DNases by low temperatures and solvents. However, since EDTA also should be effective in inactivating endogenous DNases, it was decided to investigate the wheat DNases and their inactivation.

The standard assay method for DNases as in [4] is based on the increase in absorbance at 260 nm caused by digestion of DNA. Owing to the presence of pigments in plant homogenates, this method cannot be used with concentrated preparations, and it was found to be too insensitive for use with diluted extracts. Therefore another assay was developed, based on electrophoresis of the products of digestion. This assay is unaffected by pigments, yet is sensitive and fast.

2. MATERIALS AND METHODS

2.1. *Plant material*

Seedlings of *Triticum aestivum* var. Avalon (National Seed Development Organisation Ltd.) were grown in HP3 compost (Humus Products Ltd., Bristol), for 7 days (15 h light, 9 h dark) in a greenhouse, average temperature 20°C.

2.2. *Nuclease preparation*

Green leaves were harvested by cutting at soil level, then were washed, blotted dry, weighed and chilled. They were cut into 1-cm lengths, and 1 g was homogenized in 3 ml cold 20 mM Hepes/NaOH, pH 7.6, using a pestle and mortar. The resulting homogenate was filtered through 5

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layers of butter muslin, and the filtrate (the 'crude homogenate') was collected on ice. It was found that the crude homogenate could be stored frozen (−18°C) without significant loss of nuclease activity. However, in cases where the filtrate was dialysed against 20 mM Hepes, pH 7.6, in order to remove endogenous Mg^{2+} ions, it was found that the nuclease activity was much less stable at −18°C.

2.3. *DNase assay*

The activity of a 5-μl sample of nuclease solution was assayed in a total volume of 15 μl 20 mM Hepes, pH 7.6, at 20°C. Reactions were performed in sterile 0.5 ml polypropylene microcentrifuge tubes. The substrate for nuclease activity was 100 ng λDNA (Boehringer). After incubation for 10 min, loading mix (either 30% Ficoll 400, 0.25% bromophenol blue, 90 mM Tris, 90 mM boric acid, pH 8.2, or 20% sucrose, 10% Ficoll 400, 1% bromophenol blue, 90 mM Tris, 90 mM boric acid, pH 8.2) was added to the assay mixture (1:5, v/v). For the establishment of pH optima, assay mixtures were 20 mM Hepes, 20 mM sodium acetate, with pH adjusted using acetic acid or sodium hydroxide; reactions were terminated by the addition of loading mixture containing 0.5 M $MgCl_2$, resulting in a final concentration of 80 mM $MgCl_2$. Six μl of each sample were loaded onto a 0.8% agarose (Sigma, Type I) gel for analysis by electrophoresis. The gel was run at 100 V for 30 min, in a Mini Sub DNA Cell (BioRad Labs., California), using 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, Na_2, pH 8.4. Gels were stained in ethidium bromide (final concentration 5 μg/ml) for 10 min, and were viewed on a C-63 UV transilluminator (Ultra-violet Products Inc., California).

3. RESULTS

DNase activity was found in wheat homogenates in the presence of Mg^{2+} ions (supplied in the form of $MgCl_2$), and also in the presence of the chelating agent EDTA (disodium salt), both 10 mM (table 1).

Both activities were capable of digesting a variety of double-stranded DNAs (results not shown), including high molecular weight mammalian DNA (calf thymus DNA, Sigma), bacteriophage DNA

Table 1

DNase activities of undiluted wheat homogenates

Sample	$MgCl_2$ conc. (mM)	EDTA conc. (mM)	Activity
Buffer	10	0	−
	0	10	−
DNaseI (10 μg/ml)	10	0	+
	0	10	−
Crude homogenate	10	0	+
	0	10	+

Assays were performed as described in the text. Substrate (λDNA) was either totally degraded (+), or remained undigested (−).

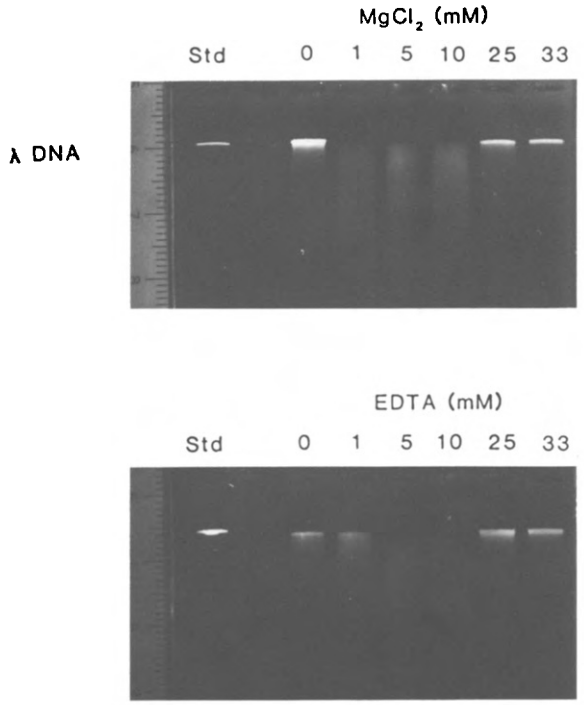

Fig.1. Effects of $MgCl_2$ or EDTA on activities of dialysed homogenates. Homogenates were dialysed against 20 mM Hepes, pH 7.6, overnight at 4°C, and were then assayed for DNase activity in the presence of various concentrations of $MgCl_2$ or EDTA, at pH 7.6, as described in the text. Homogenates were diluted 100- or 50-fold before assay with $MgCl_2$ or EDTA, respectively. Std = λDNA in buffer, without addition of homogenate.

(λDNA), and plasmid DNA (pBR322, both supercoiled and relaxed circular forms).

Homogenates were assayed at various dilutions. The EDTA insensitive activity (10 mM EDTA) was still present at 100-fold dilution, but was lost when the solution had been diluted 150-fold; the Mg^{2+} stimulated activity (5 mM $MgCl_2$) was present at 200-fold dilution, but could not be detected after a 500-fold dilution.

Homogenates which had been dialysed to remove $MgCl_2$ were found to have little DNase activity. However, activity was restored by the presence of low concentrations of either $MgCl_2$ or EDTA, the optimal concentrations being 1–10 mM and 5–10 mM respectively. At high concentrations, both $MgCl_2$ and EDTA became inhibitors of DNase activity (fig.1).

The pH optimum of each activity was determined using Hepes/acetate buffers over a range of pH values. The results (fig.2) show that both activities have pH optima at pH 7.0.

Although deproteinisation of homogenates, using phenol:chloroform (1:1, v/v), completely destroyed their DNase activities, trypsin, at a level which abolished commercial DNaseI activity, was ineffective against homogenate DNases. Diethyl pyrocarbonate (DEP) was found to be a particularly good inhibitor of both the homogenate DNase activities. These results are summarised in table 2.

Table 2

Inactivation of DNases

Sample	Treatment	Activity (+/−)	
		$MgCl_2$	EDTA
Homogenate (dil. × 2)	Trypsin	+	+
DNaseI (5 μg/ml)		−	−
Homogenate (undil.)	Phenol/	−	−
DNaseI (10 μg/ml)	chloroform	−	−
Homogenate (dil. × 10)	DEP	−	−
DNaseI (0.2 μg/ml)		−	−

Treatment conditions were: (a) trypsin, 2 mg/ml, 37°C, 30 min. (b) Phenol:chloroform, 1:1 (v/v); shaken gently for 20 min, aqueous layer recovered and re-extracted with fresh phenol:chloroform. (c) Diethyl pyrocarbonate (DEP), 1% (v/v), 0°C, 10 min. Treated samples were then assayed as described in the text, using λDNA as substrate, in the presence of 1 mM $MgCl_2$ or 10 mM EDTA.

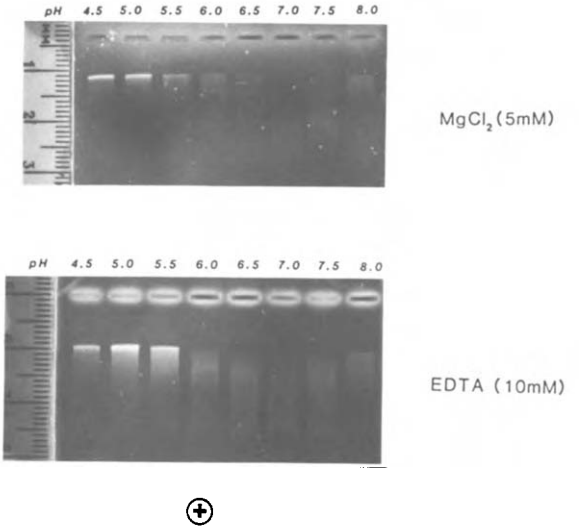

Fig.2. Variation of DNase activity with pH. Homogenates, diluted as for fig.1, were assayed for DNase activity in the presence of 5 mM $MgCl_2$ or 10 mM EDTA, in Hepes/acetate buffer at various pH values.

4. DISCUSSION

The leaves of wheat seedlings contain two types of DNase activity: one resembles DNaseI, having a requirement for Mg^{2+} and a neutral pH optimum; the other is novel, as it is activated by EDTA, and also has a pH optimum of 7.0. The activity observed with EDTA is not merely the result of inefficient chelation of Mg^{2+}, since EDTA actually increases the activity of dialysed homogenates. These activities may be characteristic of green wheat leaves, since they were not detected in wheat grain which had been germinated in darkness for 48 h [5–7]. Attempts have not yet been made to identify the locations and functions of these DNases within the cell. Clearly the chloroplast DNA is protected from degradation within the intact organelle, and is broken down only after rupture of the chloroplast membranes [2], suggesting that the DNases may be cytoplasmic in origin.

The electrophoretic method of DNase assays does not allow direct measurement of the rates of reaction, but is well suited to detecting activities which would render DNA useless for restriction analysis. Wheat leaf homogenates contain high levels of DNase activity, since even 100-fold dilu-

tions destroy high molecular weight λDNA within 10 min, at room temperature, in the presence of EDTA. It is estimated that the dilution of soluble components of leaf homogenate during a typical chloroplast isolation is of the order of 100-fold; and so residual EDTA-activated DNase is likely to lead to serious degradation of chloroplast DNA after lysis of the chloroplasts.

Our results explain why EDTA and proteolysis cannot be relied on to inactivate endogenous DNases during the isolation of DNA from wheat, and they should allow the design of more effective procedures for the recovery of high molecular weight DNA.

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