*Phytochemistry*, Vol 27, No 5, pp 1275–1279, 1988
Printed in Great Britain

0031-9422/88 $3 00 + 0 00
Pergamon Press plc

# PURIFICATION AND PROPERTIES OF ENDONUCLEASE FROM WHEAT CHLOROPLASTS, SPECIFIC FOR SINGLE-STRANDED DNA

ELŻBIETA KULIGOWSKA, DANUTA KLARKOWSKA and J W. SZARKOWSKI

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warszawa, Poland

(*Received* 2 *June* 1987)

**Key Word Index**—*Triticum vulgare*, Gramineae, wheat, chloroplast nuclease, enzyme purification, single-stranded DNA

**Abstract**—A nuclease, present in the chloroplast of wheat was purified about 150-fold The enzyme was homogeneous on polyacrylamide gel The optimal pH for denaturated DNA was 7 8 and for RNA 6.8, its $M_r$ was 29 000. The enzyme was an endonuclease degrading single-stranded DNA at a 15-fold higher rate than native DNA. The nuclease did not show an absolute requirement for added divalent cations. The enzyme degraded denaturated DNA to oligonucleotides with a phosphomonoester bond at position 5′, and RNA to 5′-OH and 3′-phosphate-terminated fragments.

The enzyme catalysed hydrolysis of synthetic polyribo- and polydeoxyribonucleotides in the following order. poly(A) > poly(U) > poly(C) > poly(G) and poly(dA) > poly(dT) > poly(dC) > poly(dG)

## INTRODUCTION

The enzymes hydrolysing DNA in higher plants known to date are nonspecific towards the sugar residue of the substrate and also degrade RNA. Nucleases have been best characterized from mung bean [1, 2], wheat seedlings [3–5] germinating pea [6, 7] and rye germs [8]. These enzymes are specific towards single-stranded DNA. According to the Wilson classification [9], they belong to the group known as nucleases type I. Although they have been used frequently in investigations on the structure of nucleic acids, the data on their biological role and subcellular localization are fragmentary and incomplete.

At present there are intensive studies on the structure of the chloroplast genome but nevertheless, there still remains much to be elucidated about the mechanism of transcription and processing of nucleic acids. A special role is played here by enzymes showing a preference for single-stranded DNA, in view of the possibility of their attacking single-stranded regions or sites with native DNA.

In the present paper a nuclease is described which was isolated from chloroplasts of wheat leaves and belonging to the group of enzymes which are nonspecific towards the sugar residue of the substrate. This enzyme, in contrast to the nucelase isolated from chloroplasts of barley leaves as described by Svachulova *et al.* [10], shows a preference for single-stranded nucleic acids

## RESULTS

### *Purity of enzyme*

The purification procedure of the enzyme is shown in Table 1 The purification was *ca* 150-fold, as compared with the specific activity in purified chloroplasts in the presence of denaturated DNA The final preparation did not show any activity towards bis-*p*-nitrophenyl-phosphate, *p*-nitrophenyl thymidine 3′- or 5′-phosphate or *p*-nitrophenylphosphate This confirmed the absence of phosphodiesterase and non-specific phosphatase activity in our preparation. The nuclease exhibits 3′-nucleotidase activity.

Polyacrylamide gel electrophoreses of the enzyme at pH values of 8.4 and 5.0, as well as in the presence of sodium dodecylsulphate at pH 7.0, (Fig 1) gave a single band indicating the homogenity of the enzyme

### *Enzyme properties*

The nuclease activity at various pH values was measured in 0.1 M acetate buffer, 0 1 citrate–phosphate buffer and 0.1 M Tris–HCl buffer. The optimal pH for denaturated DNA was 7.8 and for RNA 6 8. Relative activity of the nuclease towards native DNA, denaturated DNA and RNA is shown in Table 2 The enzyme activity towards denaturated DNA was more than 10-fold higher than that towards native DNA and more than two times higher than towards RNA These results indicate that the purified nuclease may be specific for single-stranded DNA

Studies on the influence of temperature on activity in the presence of denaturated DNA, showed an optimum activity around 50°. The effect of divalent cations and sulphydryl compounds on the hydrolysis of DNA and RNA is shown in Table 3. The data obtained indicated that $Mg^{2+}$ ions may slightly stimulate the activity towards DNA. Addition of these ions at a concentration of 10 mM resulted in only about 20% increase of activity In the case of RNase activity $Mg^{2+}$ ions at a 10 mM concentration decreased this activity to almost zero $Cu^{2+}$, $Zn^{2+}$ and $Co^{2+}$ ions at a concentration as low as 1 mM greatly depressed the enzyme activity towards DNA. $Cu^{2+}$ ions at a 5 mM concentration caused a complete loss of this activity. $Zn^{2+}$ ions at a 1 mM concentration inhibited completely RNase activity, and $Cu^{2+}$ and $Co^{2+}$ ions reduced DNase activity only slightly. When added in a 10 mM concentration they de-

Table 1 Purification scheme for wheat chloroplast nuclease

| Step | Total protein (mg) | DNAase Total activity (units/ml) | DNAase Specific activity (units/mg protein) | DNAase Purification factor | RNAase Total activity (units/ml) | RNAase Specific activity (units/mg) | RNAase Purification factor |
|---|---|---|---|---|---|---|---|
| Purified chloroplasts | 12 00 | 432 | 36 | 1 | 80 | 16 | 1 |
| Supernatant after disruption of chloroplasts | 1 28 | 2840 | 355 | 10 | 240 | 30 | 2 |
| DE-52 cellulose | 0 30 | 432 | 1440 | 40 | 3600 | 360 | 22 5 |
| Phosphocellulose | 0 048 | 264 | 5500 | 152 | 9600 | 1200 | 75 |

Fig 1 Polyacrylamide gel electrophoresis of nuclease in 10% SDS

Table 2 Wheat chloroplast nuclease activity towards denatured DNA, RNA and native DNA

| Substrate | Specific activity (units/mg protein) 1 hr | 2 hr | 3 hr | 4 hr |
|---|---|---|---|---|
| DNA denatured | 1200 | 2200 | 3300 | 5000 |
| RNA | 500 | 900 | 1250 | 1800 |
| DNA native | 100 | 200 | 280 | 350 |

Table 3 Effect of various agents on the hydrolysis of RNA and denatured DNA by nuclease*

| Agents | Concentration (mM) | Relative activity (%) DNA | RNA |
|---|---|---|---|
| Control | | 100 | 100 |
| $MgCl_2$ | 1 | 107 | 50 |
| | 10 | 119 | 4 |
| $ZnCl_2$ | 1 | 33 | 0 |
| | 10 | 10 | 0 |
| $CaCl_2$ | 1 | 100 | 84 |
| | 10 | 88 | 58 |
| $CoCl_2$ | 1 | 50 | 64 |
| | 10 | 35 | 8 |
| $CuCl_2$ | 1 | 15 | 100 |
| | 5 | 0 | 42 |
| | 1 | 10 | 80 |
| EDTA | | | |
| Mercaptoethanol | 0 5 | 68 | 85 |
| | 5 | 55 | 30 |
| Dithiotreitol | 0 5 | 74 | 81 |
| | 5 | 29 | 59 |
| PCMB | 0 5 | 96 | 95 |
| | 5 | 37 | 50 |

*Standard assay conditions Enzyme (4 $\mu$g of enzyme protein) was preincubated at 37 for 10 min

pressed its activity by about 12% $Ca^{2+}$ ions at a 10 mM concentration decreased RNase activity by 42%

The enzymatic activity towards DNA was strongly inhibited by the addition of EDTA, a 1 mM concentration of which caused a 90% inhibition, whereas with RNA the enzyme activity was inhibited only 20%

The sulphydryl reagent, *p*-chloromercuribenzoate (PCMB), had an inactivating effect on DNase and RNase, a 5 mM concentration depressed DNase activity by about 60% and RNase activity by 50% This activity was also inhibited by mercaptoethanol and dithiotreitol The latter at a 5 mM concentration depressed enzyme activity in the presence of DNA and RNA by 70 and 40% and mercaptoethanol inhibited by 50 and 70%, respectively.

The enzyme activity towards denaturated DNA (Table 4) was slightly inhibited by low NaCl concentrations. Only a concentration as high as 0.2 M caused an activity decrease of 50%.

The $M_r$ of the enzyme was determined electrophoretically on dodecyl sulphate polyacrylamide gel slabs at pH 8 3. Comparison of the mobilities of marker proteins with that of the enzyme showed a $M_r$ of 29 000 for wheat chloroplast nuclease

*Mode of degradation*

In order to determine whether the activity of wheat chloroplast nuclease was of an exonucleolytic or endonucleolytic type, denaturated DNA and RNA were digested to different degrees of acid solubilization in standard reactions and chromatographed on a Sephadex G-50 column. The enzyme initially acted in an endonucleolytic manner, producing oligonucleotides. Substances eluting at positions expected for mononucleotides were present only after much longer incubation periods.

*Identification of terminal groups in DNA and RNA digest*

The products of hydrolysis of denaturated DNA and RNA obtained by the action of nuclease were further subjected to the action of phosphodiesterase from snake venom which requires a free hydroxy group at the 3′ terminal and releases 5′-mononucleotides. The products of hydrolysis of denaturated DNA were further extensively decomposed by snake venom phosphodiesterase. Hence, it may be concluded that nuclease breaks the DNA chain yielding oligonucleotides with a free hydroxy group at the terminal C-3′ carbon. RNA hydrolysis products obtained by the action of nuclease were further separated by spleen phosphodiesterase. This indicates that the oligonucleotides formed in partial RNA hydrolysis have a free hydroxy group at the terminal carbon C-5′.

*Action of the enzyme on polyribo- and deoxyribonucleotides*

The nuclease from wheat chloroplasts showed different rates of activity on ribohomopolymers (Table 5). The enzyme degraded poly(A) at a relatively rapid rate. Poly(U) and poly (C) were hydrolysed at a slow but significant rate, whereas poly(G) was digested only to a small extent. The results in Table 5 show that, of the four deoxyhomopolymers, only poly(dA) was hydrolysed to any significant extent. Poly(dT) and poly(dC) were hydrolysed about three times more slowly than poly(dA)

Table 4 Effect of increasing sodium chloride concentration on the degradation of denatured DNA by nuclease*

| Concentration of NaCl (M) | $\Delta A_{260}$ |
|---|---|
| None | 0 28 |
| 0 05 | 0.25 |
| 0 1 | 0 21 |
| 0 15 | 0 195 |
| 0 2 | 0.155 |

*Standard assay conditions Enzyme (3 μg of enzyme protein)

Table 5 Hydrolysis of ribo- and deoxyhomopolymers*

| Substrate | $\Delta A_{260}$ | Substrate | $\Delta A_{260}$ |
|---|---|---|---|
| Poly (A) | 0 45 | Poly (dA) | 0 42 |
| Poly (G) | 0.05 | Poly (dT) | 0 18 |
| Poly (C) | 0 15 | Poly (dC) | 0 13 |
| Poly (U) | 0 22 | Poly (dG) | 0 06 |

*See Experimental for details.

## DISCUSSION

In the chloroplasts from the first leaves of wheat sprouts enzymatic activity was noted towards denaturated DNA and RNA. It is well known [11] that, when chloroplasts are isolated, a certain number of them are damaged. In the chloroplast preparations from wheat leaves obtained by us there were about 20% damaged chloroplasts. It would seem that a part of the nucleolytic activity of the purified chloroplasts (Table 1) originated from the damaged organelles. After disruption of the chloroplasts by osmotic shock further release of nucleolytically active proteins occurs, giving a 10-fold increase of activity.

Further investigations indicate that chloroplasts of the first leaves of wheat seedlings contain nuclease nonspecific towards the sugar residue substrate. The enzyme isolated from chloroplast and purified to a state of electrophoretic homogenity, hydrolyses RNA, native and denaturated DNA The rate of enzymatic hydrolysis of single-stranded DNA was more than 10 times higher than that of native DNA Similarly, as other nucleases of this type are present in higher plants, the studied enzyme also shows 3′-nucleotidase activity. Thus, in view of the noted substrate specificity it may be classified according to Wilson [9] as a plant nucleases of type I.

The $M_r$ of the investigated nuclease is 29 000, not significantly different from that of other nucleases of this type, the $M_r$ of which lie within the range 30 000–45 000. The optimum pH for the enzyme activity is, however, different from that of other nucleases of this type [9]. The nuclease from chloroplasts of the first leaves of wheat germs is most active with DNA as the substrate in a medium with pH 7.8 but with RNA it is most active at pH 6.8. Thus, in the case of chloroplast nuclease, like in that from germinating pea [6] a certain shift of pH optimum is observed towards alkaline values. It should be mentioned that the endonucleases of the unicellular algae *Chlamydomonas reinhardi* [12] and *Euglena gracilis* [13] which are specific towards single-stranded DNA show the highest activity in alkaline medium with pH 8 8 and 9 4, respectively.

Contrary to other type I nucleases, most of which require bivalent cations for maintaining their maximal activity [3, 14], chloroplast nuclease is inhibited by bivalent ions. Only $Mg^{2+}$ has a slight stimulating influence on this activity. This exclusive stimulating effect of $Mg^{2+}$ ions was observed by Svachulova *et al.* [10] in the case of barley chloroplast nuclease. Germinating pea nuclease is also activated by $Mg^{2+}$ ions [6]. The activity of the

investigated nuclease from chloroplasts of wheat leaves is strongly inhibited by EDTA An EDTA concentration of 1 mM decreases the activity by 90% Activity of barley chloroplast nuclease is inhibited by an EDTA concentration as high as 25 mM [10] Strong inhibition by EDTA was also observed in the case of endonuclease from the alga *Chlamydomonas reinhardi* [12], where a 0 05 mM EDTA concentration causes a complete loss of activity This endonuclease is at the same time activated by $Ca^{2+}$ and $Cu^{2+}$ ions Nucleases from fungi [15–17] are similarly very strongly inhibited by low EDTA concentrations.

Sulphydryl –SH donors such as mercaptoethanol and dithiotreitol in a 0 5 mM concentration at pH 7 8 have an inhibitory effect on nuclease from wheat chloroplasts Linn and Lehman [15] observed earlier that *Neurospora crassa* nuclease was similarly inhibited by low concentrations of sulphydryl compounds at alkaline pH They suggested that inhibition may be connected with reduction within the enzyme of specially sensitive disulfide bridges Most type I nucleases require the presence of sulphydryl compounds for preserving their activity at pH 4 5 The activity of the studied enzyme (unpublished data) in a medium with pH 4 5 was slightly stimulated by mercaptoethanol The same compound at pH 8 0 is inhibitory to other type I nucleases as in the case of the nuclease of wheat chloroplasts studied by us Sulphydryl reagents such as *p*-chloromercuribenzoate have an inhibitory effect on this enzyme Ethylmaleimide has a similar influence on barley chloroplast nuclease [10]

The specificity of the nuclease was established by observing its activity in the presence of natural and synthetic substrates The enzyme decomposes RNA and denatured DNA like a typical endonuclease Nevertheless, different end products were released from these substrates For denatured DNA the end products are 5′-phosphomono- and oligonucleotides with a free hydroxy group in position 3′, whereas in RNA hydrolysis leads to 3′-phosphomono- and oligonucleotides with a free hydroxy group in position 5′ The different action of wheat chloroplast nuclease on DNA and RNA distinguished it from other higher plant nucleases [9] The endonucleases from *Chlamydomonas reinhardi* [12] and *Euglena gracilis* [13] degrade RNA in a similar manner to the chloroplast nuclease [9]

It was found that ribohomopolymers are hydrolysed by the nuclease in the following sequence poly(A) > poly(U) > poly(C) > poly(G). This result agrees with the marked specificity towards bases, observed among type I nucleases Most of them hydrolyse most rapidly the bond in the neighbourhood of adenine next to uridine, and more slowly the bond near cytosine and guanosine [9] Since degradation of these compounds was investigated at pH 5 8, it would seem that higher order substrate structure had no influence on the rate of hydrolysis [18] The same sequence in hydrolysis was noted with deoxyhomopolymers The enzyme shows here too a preference for bases in the order poly (dA) > poly(dT) > poly(dC) > poly(dG) It should be noted for comparison that the mentioned observations indicate a certain similarity of wheat leaf chloroplast nuclease to chloroplast nucleases of the unicellular algae *Chlamydomonas reinhardi* [12] and *Euglena gracilis* [13]

The present results supplement the scarce data on nucleases present in chloroplasts The distinct specificity of the enzyme for single-stranded structures makes this enzyme convenient for investigations on the structure of DNA. At present further investigations are under way on the specificity of the enzyme and its natural substrates.

## EXPERIMENTAL

*Plant material* Wheat (*Triticum vulgare* var Mironowskaya) grains were surface-sterilized with 0 1% $HgCl_2$ aq soln and rinsed with $H_2O$ The grains were imbibed in $H_2O$ for 2 hr and then grown for 7 days on sterilized lignin moistened with $H_2O$, at room temp under a normal light regime

*Chemicals* Highly polymerized DNA sodium salt from calf thymus, *p*-nitrophenylthymidine 3 - or 5′-phosphate, *p*-nitrophenylphosphate, bis-*p*-nitrophenylphosphate were from Sigma Highly polymerized RNA sodium salt from yeast was produced by Sci Res Ctr Med Acad Lódz, Poland Polynucleotides were from Miles Low Molecular Weight (LMW) Calibration Kit was from Pharmacia Fine Chemicals

*Preparation of intact chloroplasts* Chloroplasts were isolated from 500 g of 7-day-old wheat leaves The procedure essentially followed the method of ref [19] but using 25 mM Tris–HCl buffer, pH 7 8 instead of 0 1 M Pi buffer, pH 6 8 Purity of the chloroplast suspension was checked in an optical microscope after staining the residual nuclei with Methylene Green

Chloroplasts free of nuclei were suspended in 10 mM Tris-HCl buffer pH 7 8 containing 1 mM $MgCl_2$ They were disrupted according to ref [10] and the supernatant was used for further purification of the enzyme

*Chlorophyll and protein concentrations* Total chlorophyll (*a* and *b*) concns were determined in 80% $Me_2CO$ by the method of ref [20]

Protein concentration was measured by the method of Lowry *et al* [21], except that the absorbance was determined at 720 nm to avoid a contribution due to chlorophyll

The percentage of intact chloroplasts was determined by the spectrophotometric method [22] The mean number of intact chloroplasts was in several preps 70–80%

*Enzyme purification* The supernatant obtained after disruption of the chloroplasts was applied to a 2 5 × 20 cm column of DE-52 cellulose equilibrated with 1 0 mM Tris HCl buffer, pH 7 8 The column was eluted with a linear gradient of 0 1–0 3 M NaCl in the same buffer at a flow rate of 6 ml 15 min Nucleolytic proteins, were eluted in the range of 0 15–0 2 M NaCl The most active fractions in the activity peak were pooled and dialysed against 10 mM Tris–HCl buffer, pH 7 8 and concd The product was then applied to a 1 5 × 15 column of phosphocellulose equilibrated with 10 mM Tris-HCl buffer, 7 8 and eluted with a linear gradient of 0 1–0 3 M NaCl in the same buffer Flow rate was 4 ml/10 min Proteins with nuclease activity were eluted in the range of 0 1–0 15 M NaCl

*Enzyme assays* RNase was assayed by measuring $A$ at $260_{nm}$ of the acid-soluble digestion products from yeast RNA according to the method described in ref [23] with the use of 0 1 M Tris–HCl buffer, pH 6 8 instead of Na-Pi The enzyme unit was defined as the amount of activity required to increase $A$ at $260_{nm}$ by 0 1 units in 1 hr DNase activity was assayed according to ref [24]

Phosphodiesterase and phosphomonoesterase were assayed on bis-*p*-nitrophenylthymidine 3′ or 5′-phosphate and *p*-nitrophenylphosphate [25]

Polyacrylamide gel electrophoresis was performed at pH 8 4 [26] and pH 5 0 [27] Electrophoresis in the presence of SDS was performed according to the method of ref [28] with 10% gel at pH 7 0 Gels were stained with 0 1% Coomassie Brilliant Blue in 50% MeOH and 7% HOAc and were electrophoretically destained in 7% HOAc and 20% MeOH

*Estimation of* $M_r$ The $M_r$ was estimated in gel slabs at pH 8.3 [29] by comparing with that of protein standards of known molecular weight

*Hydrolysis of ribo- and deoxyhomopolymers* The reaction mixture contained in the final volume of 0.4 ml 100 μg of ribohomopolymers and 4 μg of enzyme protein in 0 1 M acetate buffer, pH 5 8 Incubation was carried out at 37° for 4 hr The reaction was stopped by addition of an equal vol of 12% perchloric acid in 20 mM lanthanum acetate The samples were cooled and the non-degraded substrate was removed by centrifugation at 12 000 *g* for 30 min. After addition of 0 9 ml $H_2O$ to 0 1 ml of the supernatant fraction absorbance was measured at 260 nm

The reaction mixture contained in the final volume of 1 ml 0 2 mM of deoxyhomopolymers, 8 μg of enzyme of 0 1 M Tris–HCl buffer, pH 7 4 The mixture was incubated at 37° for 4 hr. The reaction was stopped by addition of 1 4 ml of cold 14% perchloric acid The samples were cooled and centrifuged at 1500 *g*. The absorbance of the supernatant was measured at 260 nm

*Mode of RNA and DNA degradation* The products of partial hydrolysis were analysed according to the method of ref [30], using Sephadex G-50 instead of Sephadex G-100

*Analysis of termini of degradation products* The termini produced by scission of RNA and DNA were analysed according to the method described in ref. [31]

*Protein estimation* Protein was estimated either by $A$ at $280_{nm}$ or by the method of ref [20] with trypsin as standard protein or spectrophotometrically according to the method described by Bradford [32]

*Acknowledgement*—This work was supported by the Polish Academy of Sciences within the project CPBR 3 13

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