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Processing Character of the Action of Wheat Endonucleases WEN1 and WEN2. Kinetic Parameters

L. I. Fedoreyeva[1,2] and B. F. Vanyushin[1,2]*

[1]*All Russia Research Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, ul. Timiryazevskaya 42, 127550 Moscow, Russia*
[2]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; E-mail: vanyush@belozersky.msu.ru*

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Abstract—The wheat seedling endonucleases WEN1 and WEN2 dependent on Mg^{2+}, Ca^{2+}, and S-adenosyl-*L*-methionine (SAM) and sensitive to the substrate DNA methylation status have an expressed processing action. The enzymes hydrolyze DNA at a few subsequent stages: first, they split λ phage DNA specifically at CNG-sites (WEN1) with liberation of large fragments; second, they hydrolyze these fragments to 120-140 bp oligonucleotides that finally are hydrolyzed to very short fragments and mononucleotides. Initial stages of DNA hydrolysis may proceed in the absence of Mg^{2+}, but subsequent hydrolysis stages are very strongly stimulated by Mg^{2+}. It cannot be ruled out that modulation of enzymatic activity with Mg^{2+} and probably with DNA fragments formed is associated with reorganization of the structure of eukaryotic (wheat) endonucleases with respective changes in their catalytic properties and site specificity of action. Michaelis constant value for WEN1 endonuclease on hydrolysis of methylated λ phage DNA containing Cm^5CWGG and Gm^6ATC sites is four-fold lower compared with that observed on hydrolysis of unmethylated λ phage DNA. This may indicate that affinity of WEN1 enzyme to methylated DNA is higher than that to unmethylated DNA. In the presence of SAM, the Michaelis constant for WEN2 on the DNA hydrolysis stage characterized by formation of 120-140 bp fragments is decreased, but for WEN1 it is increased by 1.5-2.0-fold. This means that SAM inhibits WEN1 but stimulates WEN2. Thus, wheat endonucleases WEN1 and WEN2 differ significantly in affinities to substrate DNAs with different methylation status, in velocities of DNA hydrolysis, and time of production of DNA fragments of similar length. It seems that the investigated plant endonucleases can hydrolyze DNA in the nucleus as well to both large and very short fragments including mononucleotides, that is, in particular, essential for utilization of cell nucleic acid material during apoptosis.

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Key words: apoptosis, endonuclease, plant, wheat, kinetics, DNA methylation, processing action, SAM

There are at least two classes of endonucleases in higher plants: Ca^{2+},Mg^{2+}-dependent and Zn^{2+}-dependent endonucleases [1]. Neutral Ca^{2+},Mg^{2+}-dependent endonucleases are found in nuclei and cytoplasm of wheat initial leaves and coleoptiles [2]. The sets and activities of these enzymes are tissue specific – they are different in nucleus and cytoplasm and change significantly with age [2, 3]. It was suggested that these enzymes are involved in nuclear DNA degradation during programmed cell death (apoptosis) in wheat coleoptiles [2]. Mg^{2+}-dependent endonuclease that takes part in apoptotic DNA degradation was found in the intermembrane space of *Arabidopsis* mitochondria [4]. Many plant endonucleases hydrolyze single- and double-stranded DNA and RNA without any expressed site-specific action [5, 6].

Ca^{2+},Mg^{2+}-dependent endonucleases (WEN1, WEN2) isolated by us from wheat coleoptiles are able to discriminate between DNA of different methylation status; the action of these enzymes is modulated by S-adenosyl-*L*-methionine (SAM) [7, 8] and histone H1 [9]. Besides, endonuclease WEN1 has expressed site-specific action: the enzyme hydrolyzes DNA at CNG-sites (between C and N residues), it prefers to split double-stranded DNA if a cytosine residue in them is methylated, and unmethylated single-stranded DNA [10]. Thus, DNA hydrolysis with these endonucleases depends on the

Abbreviations: bp, base pairs; EB, ethidium bromide; SAH, S-adenosyl-*L*-homocysteine; SAM, S-adenosyl-*L*-methionine; WEN1 and WEN2, wheat endonucleases.

* To whom correspondence should be addressed.

character of substrate DNA methylation carried out with respective DNA-methyltransferases in plant cell [11]. But unfortunately, the catalytic properties and kinetic parameters of WEN1 and WEN2 enzymes are still unknown.

The task of this work is to investigate the catalytic (kinetic) properties of WEN1 and WEN2 endonucleases isolated from wheat seedlings.

MATERIALS AND METHODS

Isolation of cytoplasmic vesicles from wheat coleoptiles. Seeds of Mironovskaya 808 winter wheat variety were germinated in darkness at 26°C as described earlier [2]. The 8-day-old seedlings were collected, washed thoroughly with tap water, and coleoptiles were separated. The isolated coleoptiles were homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.5, containing 0.4 M sucrose and bovine serum albumin (BSA, 1 mg/ml) (buffer A). The homogenate was centrifuged at 600*g* for 30 min, the sediment was discarded, and the supernatant was centrifuged at 7000*g* for 30 min. The sediment that is a vesicle fraction of coleoptile cells [12] was washed several times with buffer A without BSA.

Isolation and purification of endonucleases WEN1 and WEN2. The total endonuclease activity was extracted from the vesicular fraction [12] with 50 mM Tris-HCl buffer, pH 7.5, containing 0.8 M sucrose and 0.35 M NaCl. Endonucleases were isolated from the extract and purified by consecutive ion-exchange chromatography on DEAE-cellulose, gel filtration through Superdex 200 and Superdex 75, and hydrophobic chromatography on Toyopearl HW-50 [7, 8]. Protein concentration was determined spectrophotometrically by measuring the solution absorption values at λ = 280 nm and by Bradford's method [13].

Determination of endonuclease activity. Unmethylated (dcm^-, dam^-) and methylated (dcm^+, dam^+) λ phage DNA were used as substrates for determination of endonuclease activities of isolated enzymes. Enzyme

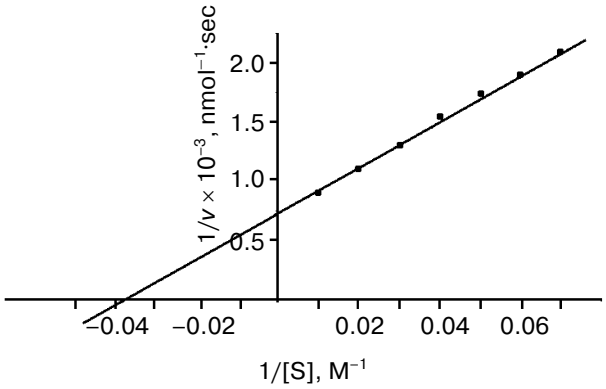


Fig. 1. Dependence of inverse velocity of reaction of methylated λ phage DNA hydrolysis with endonuclease WEN1 on inverse DNA concentration (Lineweaver–Burk plot).

(3 μl, $2 \cdot 10^{-8}$ M) was added to 1 μg (2 μl) DNA and 5 μl 50 mM Tris-HCl buffer, pH 7.5, and the mixture was incubated at 37°C for 2 h. DNA hydrolysates were fractionated and then analyzed by electrophoresis in 1.2% agarose gel.

To investigate the kinetics of DNA hydrolysis with endonucleases WEN1 and WEN2, the enzyme solution ($2 \cdot 10^{-8}$ M) was added to 0.5-3 μg DNA, and the reaction mixture was incubated at 37°C. Samples were taken from the mixture after several incubation intervals and analyzed by electrophoresis in 1.2% agarose gel. Gels were photographed in a Fujifilm FLA 3000 apparatus (Japan). Products of DNA hydrolysis were analyzed and measured quantitatively using the Gel-Pro program.

Kinetic parameters of DNA hydrolysis were calculated only for the reaction stage during which 120-140 bp oligonucleotides appear. The kinetic investigations were performed using different concentrations of substrate methylated and unmethylated phage DNA at constant concentrations of enzymes WEN1 and WEN2.

The DNA hydrolysis velocity was calculated as $v = [(\Delta I/I_0)/t] \cdot [S]$, where ΔI is a difference between optical

Kinetic parameters of actions of endonucleases WEN1 and WEN2 in DNA hydrolysis

Enzyme	Substrate		K_m, nM	$V_{max} \times 10^{-3}$, nmol/sec	$k_{cat} \times 10^{-3}$, sec^{-1}
WEN1	λ phage DNA (dcm^+, dam^+)	no SAM	20.8	1.67	0.08
		+ SAM	51.3	5.71	0.28
WEN1	λ phage DNA (dcm^-, dam^-)	no SAM	80	3.57	0.18
		+ SAM	166.7	66.7	3.33
WEN2	λ phage DNA (dcm^+, dam^+)	no SAM	100	5.5	0.37
		+ SAM	62.5	33.3	2.22
WEN2	λ phage DNA (dcm^-, dam^-)	no SAM	26.7	4.0	0.27
		+ SAM	13.1	10.5	0.7

densities of bands of respective DNA hydrolysis products (in pixels) calculated using the Gel-Pro program, I_0 is band density of initial DNA (pixels), t is time in seconds, [S] is DNA concentration (nM). V_{max} and K_m were calculated from Lineweaver–Burk plots ($1/v$ dependence on $1/[S]$) obtained using the Michaelis equation data: $v = k_{cat}[E][S]/K_m + [S]$. As an example, one of many graphs obtained is presented in Fig. 1.

The kinetic parameter values calculated from graphs of inverse reaction velocity on inverse DNA concentration (Fig. 1) are represented in the table. The mean determination error is ~10%. Fluorescence spectra were obtained using a Perkin Elmer LS 55 spectrofluorimeter (USA).

DNA of phages λ were purchased from Fermentas (Lithuania). Unlike unmethylated DNA (dcm^-, dam^-), the methylated DNA (dcm^+, dam^+) of λ phage contains 5-methylcytosine residues in Cm^5CWGG sequences and N^6-methyladenine residues in Gm^6ATC sites.

RESULTS AND DISCUSSION

Endonucleases WEN1 and WEN2 were isolated from cell vesicular fraction of aging apoptotic coleoptiles of 8-day-old etiolated wheat seedlings [7, 8]. The two enzymes were purified by similar procedures, and they were separated only by chromatography on Toyopearl HW-50. On the electrophoretic protein fractionation, the nuclease activities in gel were found in proteins with putative molecular mass ~27 (endonuclease WEN1) and ~43 kDa (endonuclease WEN2), respectively.

Endonuclease WEN1 splits predominantly methylated λ phage DNA, whereas endonuclease WEN2 hydrolyzes unmethylated λ phage DNA (Fig. 2). These enzymes are the first higher eukaryote endonucleases described that distinguish substrate DNA of various methylation status. In this property, the plant enzymes investigated are similar to some bacterial and protozoan (*Chlamydomonas*) restriction endonucleases.

Endonucleases WEN1 and WEN2 are able to hydrolyze DNA in the absence of divalent metal ions. But metal ions significantly influence the action of these endonucleases. Mg^{2+} strongly activates WEN1 and WEN2: in the presence of Mg^{2+} phage DNA was fully hydrolyzed and, therefore, respective lanes in gels with such DNA hydrolysates look empty (Fig. 2, lanes *2*). We observed earlier that WEN2 activity was stimulated with Mg^{2+} and Mn^{2+} but inhibited with Ca^{2+} [7, 8].

S-Adenosyl-*L*-methionine (SAM) significantly activates WEN2 during hydrolysis of both unmethylated (dam^-, dcm^-) and methylated (dam^+, dcm^+) λ phage DNAs. In the presence of SAM, unmethylated DNA was fully hydrolyzed practically to 120-140 bp oligonu-

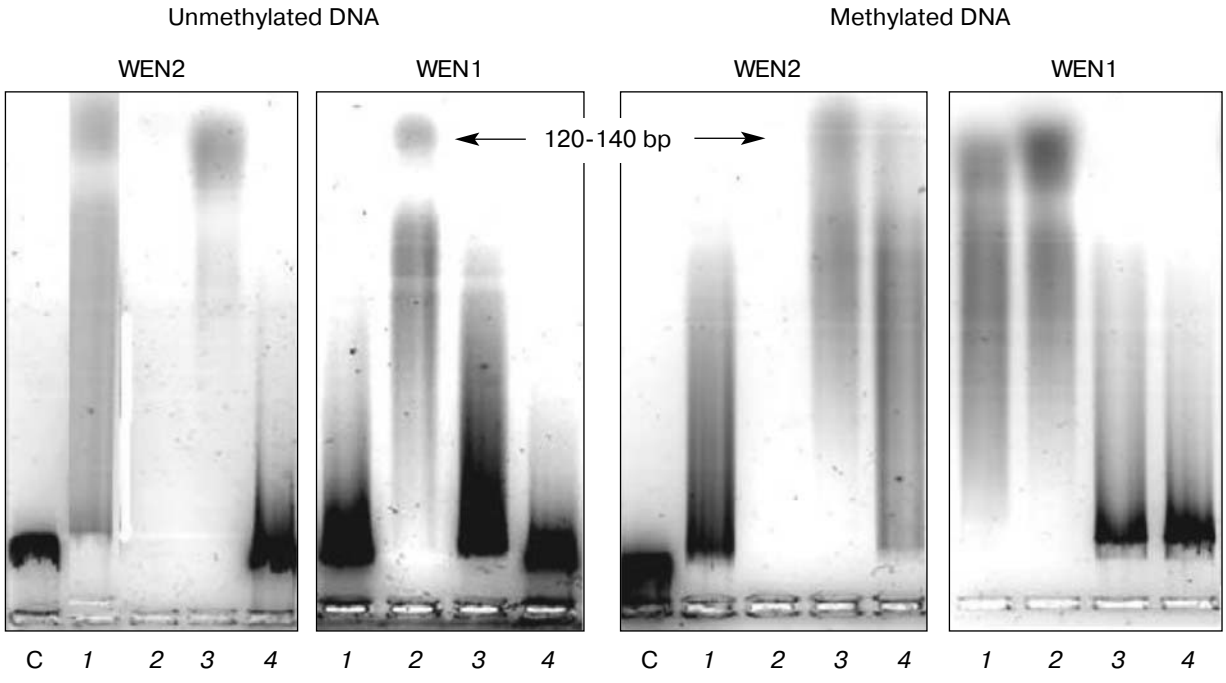


Fig. 2. Electrophoregrams (in 1.2% agarose gel) of λ phage DNA and products of its hydrolysis: *1*) products of DNA hydrolysis with endonuclease without Mg^{2+} added; *2*) products of DNA hydrolysis with endonuclease in the presence of 3 mM Mg^{2+}; *3*) products of DNA hydrolysis with endonuclease in the presence of 1 mM SAM; *4*) products of DNA hydrolysis with endonuclease in the presence of 1 mM SAH. C, control (DNA). Absence of material with adsorption of UV light (lanes *2*) means that DNA was fully hydrolyzed to low-molecular-mass products that were run out of the gel. Time of DNA hydrolysis is 2 h.

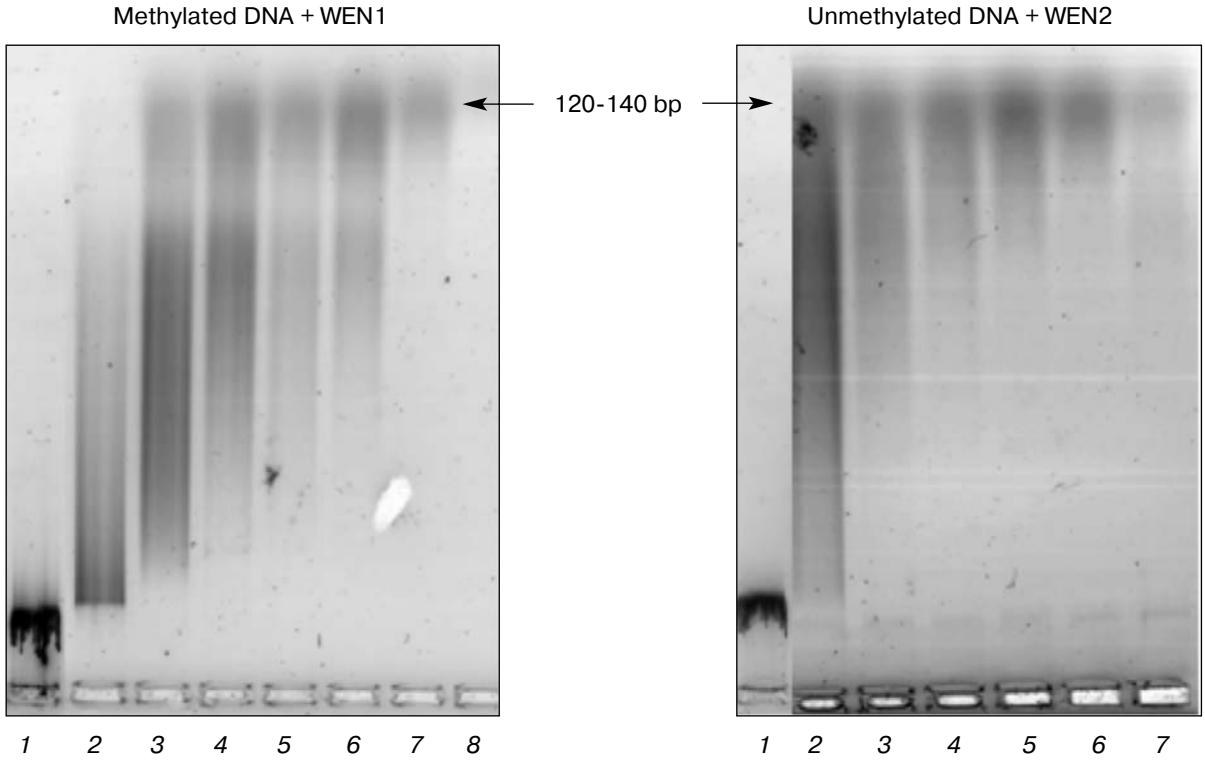


Fig. 3. Electrophoregrams (in 1.2% agarose gel) of products of the methylated and unmethylated λ phage DNA hydrolysis with endonucleases WEN1 and WEN2 in the presence of Mg^{2+} formed at various periods of hydrolysis: *1-8*) 0, 5, 10, 15, 20, 30, 45, and 60 min, respectively. Absence of material with adsorption of UV light (lanes *8*) means that DNA was fully hydrolyzed to low-molecular-mass products that were run out of the gel.

cleotides (Fig. 2). SAM modulates hydrolysis of unmethylated DNA by endonuclease WEN1; unlike stimulation of WEN2, SAM relatively weakly inhibited WEN1. But in the presence of SAM the hydrolysis of methylated DNA with endonuclease WEN1 can be significantly inhibited. S-Adenosyl-*L*-homocysteine (SAH) also modulates activity of both endonucleases [7, 8].

DNA fragments different in molecular mass were observed on analysis by agarose gel electrophoresis of DNA hydrolyzed for different periods with wheat endonucleases at constant temperature (Fig. 3). At initial stage of hydrolysis, DNA was split to large fragments that were hydrolyzed then to 120-140 bp oligonucleotides. Then these DNA hydrolysis products were degraded to very short fragments including mononucleotides. Deoxyribomononucleotides were detected in phage DNA hydrolysates by chromatography on C18 column in a linear (0-100%) gradient of acetonitrile.

More or less discrete sets of the DNA hydrolysis products originating and changing subsequently at different intervals of hydrolysis may be due to processing character of wheat endonuclease action at the different hydrolysis stages. For example, endonucleases G from various sources are able to process primers for replication of mitochondrial DNA. Enzyme endoG is an apoptotic protein that liberates from mitochondria and participates in caspase-independent nuclear DNA fragmentation, and it is important for normal cell proliferation [14].

Interaction of enzymes with substrate DNA was judged by changes in the fluorescence spectra of DNA–ethidium bromide (EB) complexes treated with endonucleases. It is known that fluorescence of DNA–EB complexes is markedly quenched on DNA fragmentation that may occur and on DNA hydrolysis with endonucleases.

In fact, gradual fluorescence quenching of complex methylated DNA–EB occurs with time during hydrolysis of its DNA by endonuclease WEN1 (Fig. 4). A similar picture was observed on hydrolysis of unmethylated DNA–EB complex by the same enzyme, but the decrease in fluorescence was by twofold less compared with that observed on hydrolysis of methylated DNA–EB complex. On hydrolysis of EB–methylated DNA and EB–unmethylated DNA complexes with endonuclease WEN2, fluorescence quenching was almost not observed. This means that mechanisms of endonucleolytic DNA hydrolysis by plant endonucleases WEN1 and WEN2 are different. It can be suggested that in the beginning the endonuclease WEN1 unwinds the DNA double helix and only after that it splits DNA into long oligonucleotides.

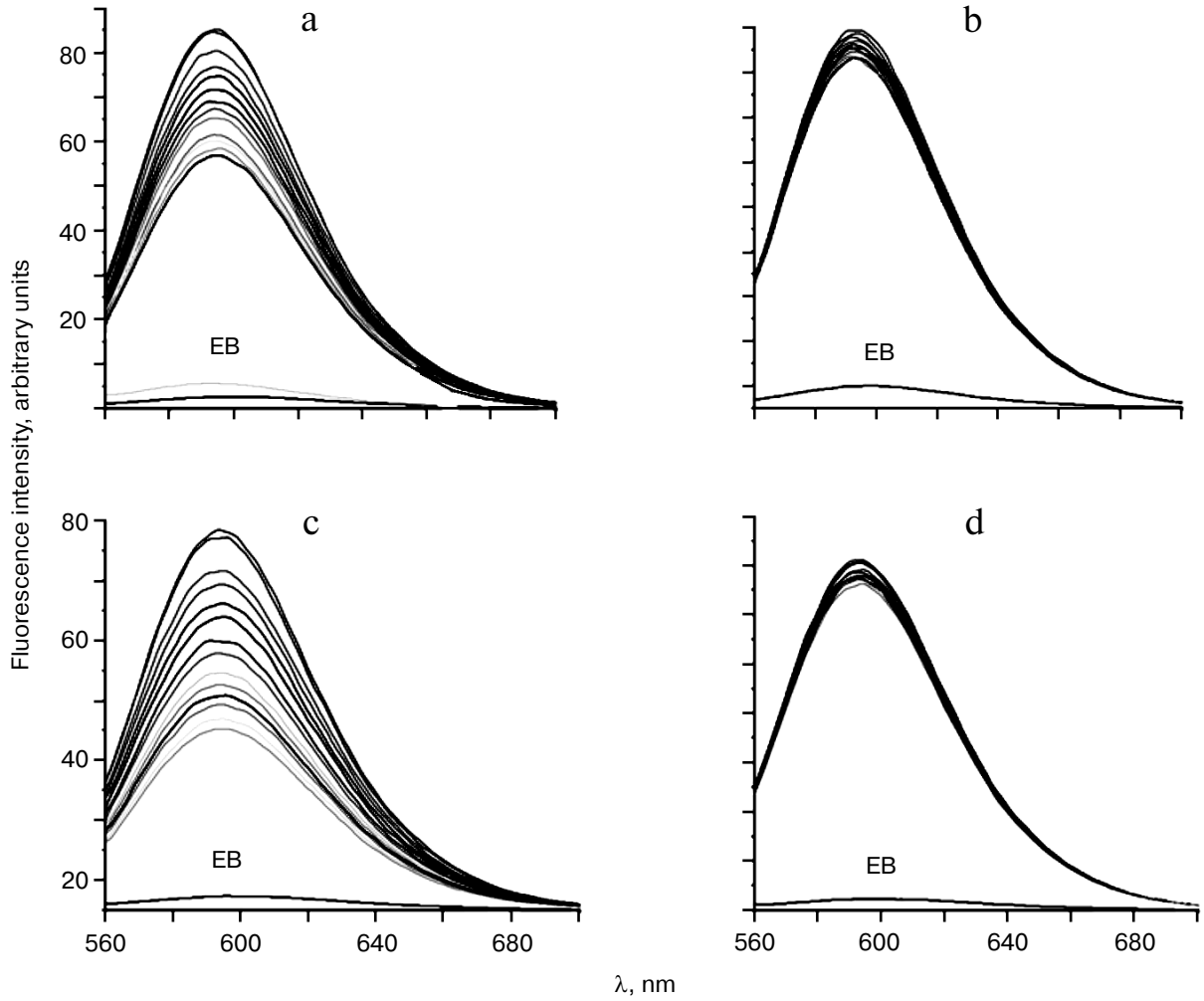


Fig. 4. Dynamics of fluorescence quenching (on excitation with λ = 540 nm light) of ethidium bromide (EB)–λ phage DNA–endonuclease complexes. a) Complex EB–unmethylated DNA–WEN1; b) complex EB–unmethylated DNA–WEN2; c) complex EB–methylated DNA–WEN1; d) complex EB–methylated DNA–WEN2. Upper curve on each figure is fluorescence of complex EB–DNA, other curves (downstream) are fluorescence of complex EB–DNA–enzyme (spectra are taken with 2 min intervals).

Endonuclease WEN2 seems to hydrolyze double-stranded DNA without its unwinding; therefore, decrease in the fluorescence intensity due to disruption of DNA–EB binding is not significant.

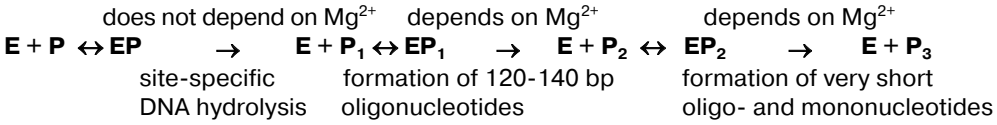
Site-specificity of wheat endonuclease action has been displayed at the initial stage of DNA hydrolysis. Endonuclease WEN1 recognizes CNG sites [10]. The substrate specificity of endonuclease WEN2 is still unknown. On the initial stage of DNA hydrolysis, the presence of metal ions is not obligatory, and efficiency of DNA hydrolysis depends only on enzyme specific activities.

Further hydrolysis of DNA fragments formed results in appearance of 120-140 bp oligonucleotides. This reaction stage depends on metal ions and other modulators of endonuclease activity.

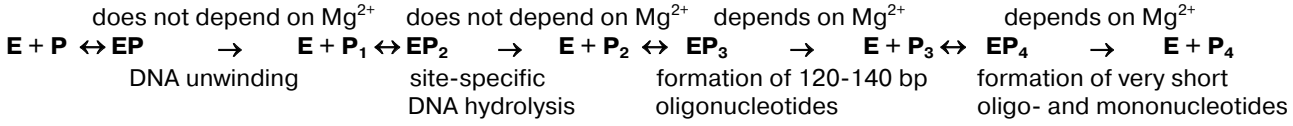
The Michaelis constant for hydrolysis of unmethylated DNA with endonuclease WEN1 is 80 nM. For hydrolysis of methylated DNA this constant is by about four-fold less – 20.8 nM. This may mean that affinity of endonuclease WEN1 to methylated DNA is much higher than to unmethylated DNA. The Michaelis constant for hydrolysis of methylated DNA with endonuclease WEN2 is 100 nM, but for hydrolysis of unmethylated DNA it is 26.7 nM.

Endonuclease WEN1 seems to have higher affinity to methylated DNA than endonuclease WEN2 to unmethylated DNA. WEN1 has higher velocity of initial hydrolysis of unmethylated DNA compared with that of WEN2 on hydrolysis of methylated DNA. This corresponds to our data on the electrophoretic analysis of DNA hydrolysis products in agarose gel showing that endonuclease WEN1 prefers to hydrolyze methylated DNA, but endonuclease WEN2 preferentially splits unmethylated DNA.

The catalytic constant k_{cat} that depends on enzyme concentration and determines the number of enzyme



Scheme 1



Scheme 2

turnovers in time is one of the fundamental characteristics of enzymatic reaction. The k_{cat} values for plant endonucleases WEN1 and WEN2 were calculated using the equation: $k_{cat} = V_{max}/[E]$. The results are presented in the table. It can be seen that corresponding catalytic constants for endonuclease WEN2 are higher than that for endonuclease WEN1, this meaning that for formation from DNA of equal length oligonucleotides endonuclease WEN2 should perform more turnovers than endonuclease WEN1.

The kinetic parameters of DNA hydrolysis with endonucleases WEN1 and WEN2 in the presence of SAM were determined as already described and are also presented in the table. In the presence of SAM, the Michaelis constant for endonuclease WEN2 at the stage of DNA hydrolysis with formation of 120-140 bp oligonucleotides is decreased by 1.5-2.0-fold. This corresponds to our observations that in the presence of SAM the concentration of these DNA fragments was increased. Unlike WEN2, the Michaelis constant for endonuclease WEN1 in the presence of SAM increased by twofold and more, and the endonuclease activity decreased respectively.

Analysis of the hydrolysis products using the Gel-Pro program after 1 h of DNA hydrolysis showed that the concentration of 120-140 bp fragments decreased with incubation time. The enzyme splits these oligonucleotides formed into very short fragments and mononucleotides (these hydrolysis products were run out of the gel and not seen on the electrophoregram, but they were detected by HPLC on a C18 column in a linear concentration gradient of acetonitrile). Thus, the character (length) of the DNA hydrolysis products depends on the hydrolysis time. At the final stage of DNA hydrolysis, the studied plant endonucleases seem to be able already to act as exonucleases. It is known that some mammalian endonucleases possess exonuclease activity [15]. The final DNA hydrolysis stage with the investigated plant endonucleases depends on metal ions, and it goes only after formation of oligonucleotides.

Based on these data, we suggest the following presumable schemes of DNA hydrolysis with endonuclease WEN2 (Scheme 1) and endonuclease WEN1 (Scheme 2), where E is endonuclease, P is initial ligand (DNA), EP is intermediate complex endonuclease–DNA, and P_1, P_2, P_3, and P_4 are products of respective DNA hydrolysis stages.

Thus, wheat endonucleases WEN1 and WEN2 are processing enzymes, and they are significantly different in affinity to substrate DNA with different methylation status, DNA hydrolysis velocities, and periods of formation of similar length DNA fragments. These enzymes seem to have exonuclease activities at the later stage(s) of DNA hydrolysis. At the earliest stages of DNA hydrolysis, the enzymes seem to have site-specific action with recognition of CNG sites in particular. After hydrolysis at these sites the site specificity of endonuclease action disappears, and they are able to split finally the fragments formed practically to mononucleotides. Unfortunately, we do not know how these enzymes work in the cell. Nevertheless, the expressed processing character of their observed action may have biological sense. It may be that after initial selective site-specific DNA hydrolysis to some domains, the DNA fragments that appear serve as some signals for reorganization of chromatin and enzyme structures and for changing of properties of the endonucleases that result in elimination of their narrow site specificity but induce their ability to fully hydrolyze DNA. In fact, the reorganization of structure and the changes in activity of endonucleases during DNA hydrolysis seem really to take place as we, in fact, observe two types of changing nuclease activity in these enzymes, one of which is metal-dependent and other being metal-independent.

The investigated plant endonucleases seem to be able to split DNA as well in the nucleus to both large fragments and very short oligo- and mononucleotides. Therefore, they may participate in utilization of the nucleic acid material, for example, during apoptosis.

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