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Wheat Coleoptile Endonuclease Wen2 Is Dependent on S-Adenosyl-L-methionine and Sensitive to DNA Methylation Status

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

[1]*Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, ul. Timiryazevskaya 42, 127550 Moscow, Russia; fax: (495) 977-0947; E-mail: bfvan@iab.ac.ru*
[2]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-3181; E-mail: vanyush@belozersky.msu.ru*

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Abstract—Endonuclease WEN2 with an apparent molecular mass 21.5 kD was isolated from subcellular vesicular fraction obtained from aging apoptotic coleoptiles of 8-day-old etiolated wheat seedlings and partially characterized. Similar to wheat endonuclease WEN1 of the same origin described earlier, the WEN2 enzyme is a neutral Ca^{2+},Mg^{2+},Mn^{2+}-dependent endonuclease. Both enzymatic activities were found also in nuclei from the same cells. Mn^{2+} activates WEN2 more efficiently than Mg^{2+} or Ca^{2+}. High ionic strength, Zn^{2+}, and EDTA inhibit the enzyme completely. In the presence of Mg^{2+}, elevated WEN2 activity was observed at pH between 5.5 and 7.7 and at 37°C, and without Mg^{2+} added it was observed in narrower pH range (from pH 6.8 to pH 7.7). The enzyme is active even at high temperature (65°C). WEN2 splits preferentially unmethylated, but WEN1 – methylated λ phage DNA. Double-stranded DNA is a preferential substrate to be hydrolyzed with WEN2. S-Adenosyl-*L*-methionine (SAM) significantly activates endonuclease WEN2 (the optimal SAM concentration is 0.3 mM). Contrary to strong stimulating action on WEN1, the competitive inhibitors of the DNA methylation reaction (SAM analogs S-adenosyl-*L*-homocysteine and S-isobutyladenosine) at concentration 0.3 mM increase WEN2 activity slightly. It is suggested that WEN2 may take part in apoptotic DNA degradation. Thus, in plants there are endonucleases that recognize methylation status of substrate DNAs and are modulated by the methyl group donor, SAM, in different fashions. Therefore, all this may indicate the presence of a restriction–modification (R–M) system in higher plants.

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Key words: apoptosis, S-adenosyl-*L*-methionine, aging, DNA methylation, endonuclease, mitochondria, nucleus, plant, wheat

Endonucleases play an important role in many genetic processes including replication, DNA repair, and recombination; they are involved in the key stages of apoptosis and, in particular, terminal ones. At least two classes of endonucleases have been found in higher plants: Ca^{2+}-dependent and Zn^{2+}-dependent endonucleases [1-5]. Ca^{2+}-dependent endonucleases are activated by Ca^{2+} as well as often by Mg^{2+} and Mn^{2+}, but all of them are strongly inhibited with Zn^{2+} [1, 3]. These endonucleases are most active at neutral pH values and preferentially hydrolyze single-stranded DNA compared with double-stranded DNA and RNA. Active Ca^{2+}-dependent endonucleases were detected in the nuclei of apoptotic plant cells [1]. Ca^{2+},Mg^{2+}-dependent endonuclease was found in the nuclei of wheat nucellus cells, this enzyme seemingly taking part in the apoptotic DNA fragmentation; it at least carries out the internucleosomal DNA degradation in nuclei isolated from human cells [6]. Mg^{2+}-dependent endonuclease was found in the intermembrane zone of mitochondria in *Arabidopsis thaliana* plants; it was suggested that this enzyme can be one of the terminal executors of apoptosis [7], being responsible for internucleosomal fragmentation and subsequent DNA degradation.

Zn^{2+}-dependent plant endonucleases are localized predominantly in cellular vacuoles; they are represented mainly by monomer glycoproteins with molecular mass

Abbreviations: SAH) S-adenosyl-*L*-homocysteine; SAM) S-adenosyl-*L*-methionine; SiBA) S-isobutyladenosine; TBE) Tris-borate buffer with EDTA.

* To whom correspondence should be addressed.

33-44 kD, hydrolyze RNA, single- and double-stranded DNA, have maximal activity at pH 5.0-6.5 [2, 4], and are rather similar in properties to fungal endonucleases S1 and P1 [5]. Unfortunately, the structure, site specificity action, nature and functional role of their potential modulators, and the mechanisms of action of plant endonucleases are not adequately explored.

We recently isolated the first eukaryotic Ca^{2+},Mg^{2+}-dependent endonuclease WEN1 (wheat endonuclease 1) from aging wheat coleoptiles that recognizes substrate DNAs different in their methylation status and hydrolyzes them in a different fashion [8]. Besides, activity of this enzyme increases in the presence of S-adenosyl-*L*-methionine (SAM), S-adenosyl-*L*-homocysteine (SAH), and S-isobutyladenosine (SiBA). This is only the case when it was established that eukaryotic endonucleases similarly to some bacterial restriction endonucleases can be modulated with SAM (a universal donor of methyl groups in organisms) and its derivatives that seem to be allosteric effectors of an enzyme. Therefore, a new principle (type) of regulation of activity of eukaryotic endonucleases has been discovered.

In the present article, we demonstrate that this event is not a unique one, and it may be quite common for other eukaryotic endonucleases. We describe here the endonuclease WEN2 (wheat endonuclease 2) isolated from aging wheat coleoptiles; this enzyme differs from WEN1 isolated earlier but it has a common property—WEN2, like WEN1, recognizes substrate DNAs with different methylation status and is modulated with SAM. According to our preliminary data, endonucleases of this type are present in other eukaryotes as well.

MATERIALS AND METHODS

Isolation of nuclei and cytoplasmic vesicles. Seeds of winter wheat Mironovskaya 808 variety were germinated in darkness in a thermostat at 26°C, and then plants were grown under the same conditions for eight days. Seedlings were thoroughly washed with water, and coleoptiles were isolated (an intense apoptosis in coleoptiles proceeds at this developmental stage under the described growth conditions [9, 10]). Coleoptiles were homogenized at 4°C in homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.4 M sucrose, 1 mg/ml BSA; 5 ml buffer per g plant tissue). The homogenate was filtered through 4-layer gauze and 1-layer Miracloth. Nuclei were isolated from homogenate by 15 min centrifugation at 600*g*. The supernatant was then centrifuged for 15 min at 3000*g* to obtain the pellet with vesicles. Pellets (fractions) containing nuclei or vesicles were washed with homogenization buffer without BSA.

Extraction of total protein from nuclei and vesicles. Pellets containing nuclei or vesicles were suspended in buffer (four volumes) containing 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride and incubated in the cold for 15 min with mixing. The suspensions were centrifuged for 10 min at 12,000*g*, and the supernatants obtained were used for determination of endonuclease activity by electrophoresis in polyacrylamide gel with embedded (melted) calf thymus DNA.

Isolation and purification of WEN2 endonuclease. Endonuclease activity was extracted from vesicular fraction with buffer containing 50 mM Tris-HCl, pH 7.5, 0.8 M sucrose, and 0.35 M NaCl. To decrease the ionic strength of the solution, the extract was diluted 10-fold with water and applied onto a column with DEAE-cellulose. The column was then washed with 10 mM Tris-HCl buffer, pH 7.5, and proteins were eluted with a NaCl linear concentration gradient (from 0 to 0.5 M) in the same buffer. The peak endonuclease activity was eluted at the salt concentration of 0.22-0.25 M.

To concentrate the fraction with nuclease activity, it was initially diluted by 10-fold with 10 mM Tris-HCl-buffer, pH 7.5, and applied onto a column with DEAE-cellulose, the column was washed with the same buffer, and the protein was eluted with 0.5 M NaCl. The protein fraction obtained was then additionally purified and desalted by gel filtration through Superdex-200 and Superdex-75 in 10 mM Tris-HCl-buffer, pH 7.5.

The protein concentration was determined spectrophotometrically by measuring the absorption value at $\lambda = 280$ nm and by Bradford's method [11]. Molecular mass of proteins was evaluated by in SDS-PAGE in 12.5% gel according to Laemmli's procedure [12]. Phosphorylase B (94 kD), BSA (67 kD), ovalbumin (43 kD), carboanhydrase (30 kD), soybean trypsin inhibitor (20 kD), and lactalbumin (14.4 kD) were used as molecular mass markers.

Determination of endonuclease activity. Total wheat DNA, unmethylated λ phage DNA (dcm^-, dam^-), and methylated λ phage DNA (dcm^+, dam^+) were used as substrates for determination of endonuclease activity. These phage DNAs are different only in the nature of their hosts: they are isolated from λ phage particles grown in cells of *Escherichia coli* (*dam*, *dcm*)$^-$ or *E. coli* (*dam*, *dcm*)$^+$, respectively. Contrary to unmethylated (*dam*, *dcm*)$^-$, the methylated phage DNA (*dam*, *dcm*)$^+$ contains residues of 5-methylcytosine in Cm^5CWGG sequences and of N^6-methyladenine in Gm^6ATC sites. Enzyme (0.25-0.5 μg) and 5 μl 50 mM Tris-HCl buffer, pH 7.2, were added to 1 μg substrate DNA, and the total volume of the reaction mixture was brought up to 10 μl with addition of bidistilled water. The reaction mixture was incubated at 37°C for 2 h (if not indicated otherwise), then buffer (2 μl) containing 10% glycerol, 0.01% bromophenol blue and xylene cyanol in TBE buffer was added, and the mixture was applied on 0.7% agarose gel in TBE buffer with 0.0005% ethidium bromide and electrophoresed. After electrophoresis, the products of the DNA hydrolysis were visualized in UV-light.

Direct detection of the endonuclease activity in polyacrylamide gel. Commercial calf thymus DNA (0.2 mg/ml) was added to 12.5% polyacrylamide gel during its preparation. DNA dissolved in bidistilled water was preliminarily precipitated twice from 70% ethanol. To remove SDS, the gel after electrophoresis was washed in distilled water for 2 h with mixing, the washing waters being changed in each 15-20 min (the first gel washing was performed with 0.5% Triton X-100 solution). After washings, the gel was placed in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$ and incubated at 37°C overnight. Ethidium bromide was then added to the buffer with gel (to concentration 0.0025%), and the products of DNA hydrolysis were visualized in UV-light (before and after 15 min incubation).

RESULTS AND DISCUSSION

Along with endonuclease WEN1 described earlier [8], one more endonuclease named as WEN2 with an apparent molecular mass 21.5 kD was clearly detected in the cytoplasmic vesicles (they are characteristic for apoptotic cells) and in nuclei isolated from coleoptiles of the 8-day-old etiolated wheat seedlings (Fig. 1). Thus, in apoptotic cells the enzyme WEN2 was observed both in vesicles and nuclei, its activity in nuclei being higher than

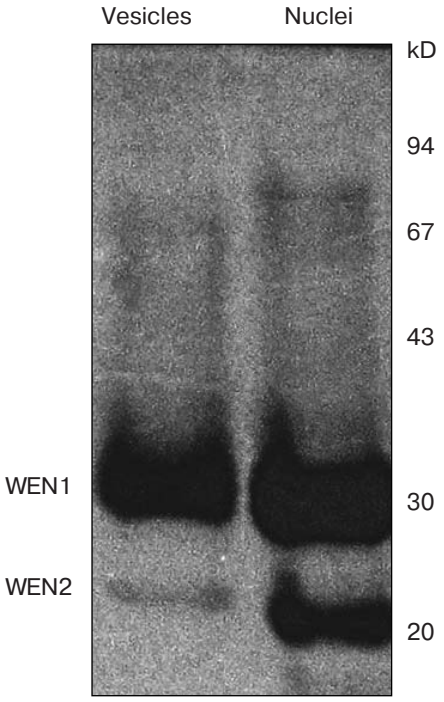

Fig. 1. The in-gel endonuclease activity of total protein extracts from vesicular and nuclear fractions of aging wheat coleoptiles in 12% polyacrylamide gel with embedded (melted) calf thymus DNA.

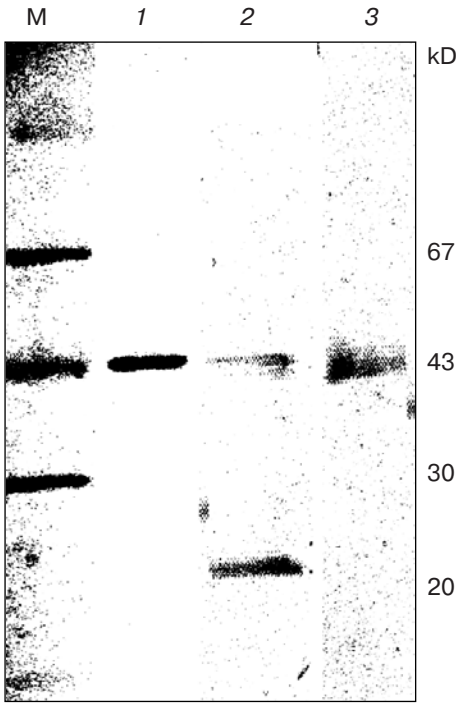

Fig. 2. Electrophoresis in polyacrylamide gel of the purified protein fraction with endonuclease activity: *1*) endonuclease WEN2 under denaturing conditions; *2*) endonuclease WEN2 under non-denaturing conditions; *3*) 21.5 kD protein (taken from *2*) now under denaturing conditions; M) markers.

in vesicles. Nevertheless, our attention was drawn to investigation of the vesicular enzyme as it was done earlier with WEN1 [8]. Because vesicles of the apoptotic cells contain mainly intact mitochondria with intense synthesis of mtDNA [9, 10], one could suggest that WEN2 found in the vesicle fraction is located mainly in mitochondria and seems to be associated with mtDNA replication. Detection of strong enzymatic activity (with mobility in gel similar to WEN2) in nuclei (Fig. 1) isolated from coleoptile cells with strong internucleosomal DNA fragmentation [10] may indicate that enzyme WEN2 or its analog seems to participate in the apoptotic degradation of nuclear DNA. Unfortunately, we do not know yet whether vesicular enzyme WEN2 is identical to the respective endonuclease activity (having the same mobility in gel) observed in the nuclear extract (Fig. 1). According to our preliminary data, the WEN2 activity (in contrast to WEN1) was not detected in isolated free non-vesicular mitochondria of aging coleoptiles of 8-day-old wheat seedlings. But we cannot rule out that WEN2 may still be present in vesicular mitochondria. If WEN2 is associated with induction and replication of mtDNA, as we have already suggested, the vesicular mitochondria are the right places for this enzyme because an intense synthesis of mtDNA and replication of mitochondria proceed in vesicles, while free non-vesicular mitochondria

do not seem to replicate significantly in the same apoptotic cells.

Endonuclease WEN2 was extracted from the vesicle fraction with 50 mM Tris-HCl buffer, pH 7.5, containing 0.8 M sucrose and 0.35 M NaCl, and sequentially purified by ion-exchange chromatography on DEAE-cellulose and gel filtration through Superdex-200 and Superdex-75. On the non-denaturing electrophoresis in isolated and purified protein fraction, the endonuclease activity was detected in two polypeptides with apparent molecular mass 21.5 and 43 kD, respectively (Fig. 2, lane *2*). Only one polypeptide with apparent molecular mass 43 kD was found on SDS-PAGE under denaturing conditions (Fig. 2, lane *1*). To detect possible subunit organization of the enzyme, the protein with molecular mass 21.5 kD was extracted from non-denaturing gel and analyzed by SDS-PAGE under denaturing conditions; its molecular mass was 43 kD (Fig. 2, lane *3*). It seems that in the "native" state the endonuclease WEN2 does exist as a monomer with molecular mass 21.5 kD.

For investigation of endonuclease activities, we used λ phage DNA (dam$^-$, dcm$^-$) unmethylated and λ phage DNA (dam$^+$, dcm$^+$) methylated in sites Gm^{6}ATC and Cm^{5}CWGG. Judging by analysis of the DNA hydrolysis products (Fig. 3), endonuclease WEN2 prefers unmethylated λ phage DNA as a substrate. On the contrary, endonuclease WEN1 isolated earlier from vesicular fraction of aging wheat coleoptiles preferentially hydrolyzed methylated λ phage DNA [8].

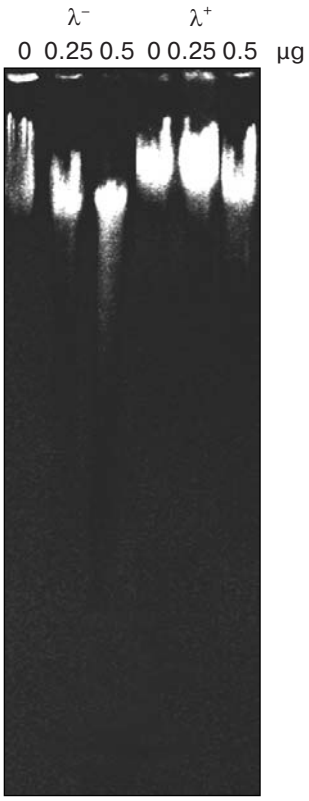

Fig. 3. Products of hydrolysis of unmethylated (λ$^-$) and methylated (λ$^+$) λ phage DNA with purified endonuclease WEN2 at pH 7.5 and 37°C in the absence of added Mg^{2+}, depending on enzyme concentration (controls).

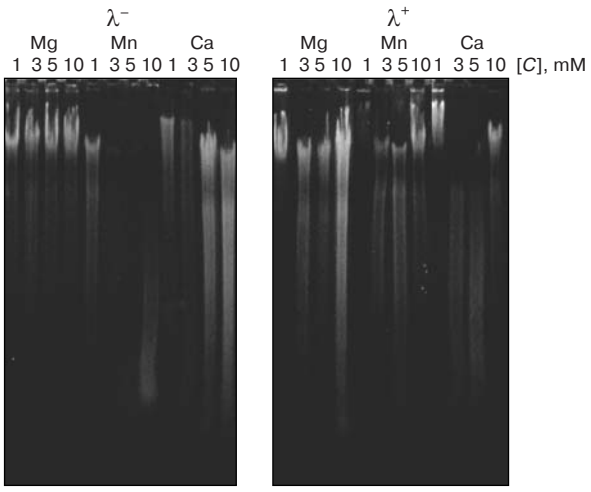

Fig. 4. Influence of the divalent metal ions on the endonuclease activity of WEN2 (0.25 μg enzyme). Hydrolysis of unmethylated (λ$^-$) and methylated (λ$^+$) λ phage DNA.

WEN1 and WEN2 are the first eukaryotic endonucleases described that recognize the methylation status of substrate DNAs. This phenomenon is common for highly specific bacterial and protozoan (*Chlamydomonas*) restriction endonucleases that belong to the restriction–modification system [13]. Thus, the restriction–modification system or some its elements seem to exist in plants.

We have studied the influence of divalent metal ions on the nuclease activity of WEN2. Zn^{2+} always fully inhibited the enzyme activity even at low concentrations (less than 0.5 mM) (not shown). Thus, wheat endonuclease WEN2 explicitly is not a Zn^{2+}-dependent endonuclease, but it is a Ca^{2+},Mg^{2+}-dependent enzyme. Ca^{2+}, Mg^{2+}, and Mn^{2+} strongly activated the endonuclease; in the presence of these ions, WEN2 hydrolyzed both unmethylated (dam$^-$, dcm$^-$) and methylated (dam$^+$, dcm$^+$) λ phage DNAs (Fig. 4). WEN2 activity significantly depends on the concentration of metal ions. Optimal Mg^{2+} concentration in the reaction mixture is 3 mM. It is curious that Mn^{2+} activates the enzyme more efficiently than Mg^{2+} or Ca^{2+}. It can be seen (Fig. 4) that Mn^{2+} stimulates DNA hydrolysis with endonuclease WEN2 at lower concentrations than Mg^{2+} and Ca^{2+}. In the presence of Mn^{2+} (5 mM and less), WEN2 splits unmethylated λ phage DNA down to low molecular weight oligonucleotides (120-140 bp). The optimal Mn^{2+} concentration is even lower (3 mM); at this Mn^{2+} concentration, unmethylated λ phage DNA was hydrolyzed fully to shorter fragments that migrate quickly during electrophoresis and practically run out of the gel (not

seen in the gel) (Fig. 4). Therefore, in fact, WEN2 seems to be more reasonable to consider as a Mn^{2+}-dependent enzyme. Anyway, it can be clearly seen that in all cases including the presence of other metal ions, WEN2 hydrolyzed unmethylated DNA much stronger than methylated DNA. Relatively high concentrations of metal ions in the reaction mixture inhibit slightly the enzyme activity. Addition of EDTA to the medium results in full inhibition of WEN2 activity (not shown). Increasing ionic strength by NaCl or KCl adding (10-100 mM) to reaction mixture also fully inhibits the enzyme. Subsequent addition of divalent metal ions to the medium with high ionic strength does not reactivate WEN2 (not shown).

The dependence of reaction of the unmethylated (dam^-, dcm^-) λ phage DNA hydrolysis with endonuclease WEN2 at the optimal Mg^{2+} concentration (3 mM) on temperature and incubation time is presented in Fig. 5. We determined the time needed for full DNA hydrolysis with endonuclease at 25, 37, and 65°C, respectively. At 25°C DNA was fully hydrolyzed in 90 min. At 37°C, DNA hydrolysis was accomplished in 75 min with formation of shorter fragments. Thus, temperature such as 37°C is optimal for hydrolysis of unmethylated double-stranded DNA with endonuclease WEN2 to relatively low molecular mass fragments (oligonucleotides). The degree of DNA hydrolysis at 65°C diminishes significantly. At this temperature, the hydrolysis proceeds more quickly (it was finished in 30 min), but the DNA was hydrolyzed only to relatively long fragments. We suggest that decrease in degree of DNA hydrolysis at the elevated temperature (65°C) is mainly associated not with decrease in proper endonuclease activity, but with partial denaturation of substrate DNA and appearance of single-stranded stretches in it. After 2 h incubation of WEN2 at 65°C, it hydrolyzed unmethylated DNA at 37°C with formation of practically the same oligonucleotide set as that produced by "warmed" enzyme. Products of the λ phage DNA hydrolysis at 37°C with a preliminary enzyme incubated at 65°C were similar to those obtained by hydrolysis at 65°C (Fig. 5). This means that endonuclease WEN2 prefers the double-stranded unmethylated λ phage DNA as a substrate to be hydrolyzed, and the partial thermal DNA denaturation seems to diminish the availability of the substrate to WEN2.

Data on dependence of the WEN2 endonuclease activity on pH values are presented in Fig. 6. Unmethylated λ phage DNA was used as a substrate. Reaction of DNA hydrolysis was performed in the presence of 3 mM Mg^{2+}. We observed that under these conditions WEN2 was most active at pH 5.5-7.7. In the absence of Mg^{2+}, the enzyme was most active in a narrower pH interval (from 6.8 to 7.7). Thus, WEN2 belongs to the group of so-called neutral Ca^{2+},Mg^{2+}-dependent plant nucleases.

It is well known that enzymatic DNA methylation is carried out by specific cytosine and adenine DNA-methyltransferases that use SAM as a donor of methyl groups. As far as endonuclease WEN2 from wheat seedlings is sensitive to the presence of methylated

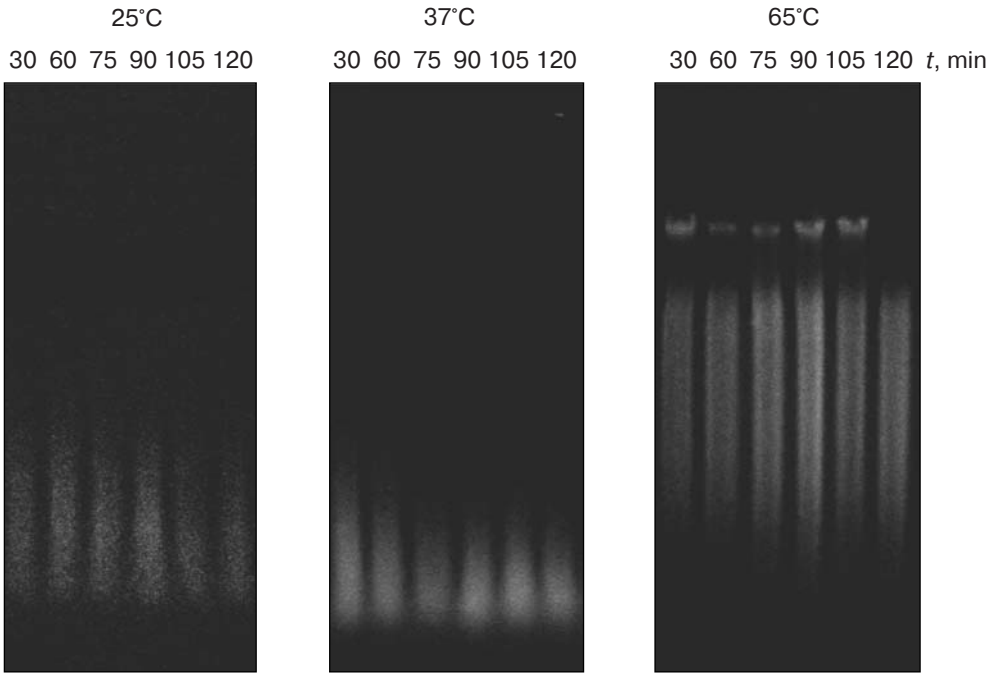

Fig. 5. Influence of temperature on activity of endonuclease WEN2 (0.5 μg). Substrate, unmethylated λ phage DNA.

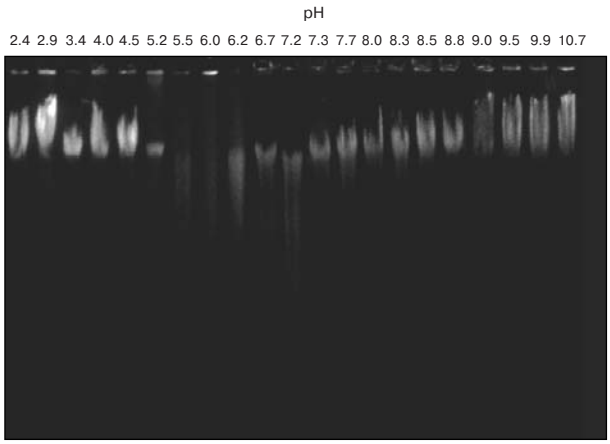

Fig. 6. Dependence of WEN2 (0.25 μg) activity on pH. Substrate, unmethylated λ phage DNA.

nucleotide residues in substrate DNAs, we tried to investigate the influence of SAM on the endonucleolytic activity of the enzyme (Fig. 7). It can be seen that SAM strongly activates WEN2 both on hydrolysis of unmethylated (dam^-, dcm^-) and methylated (dam^+, dcm^+) λ phage DNA. The optimal SAM concentration is 0.2-0.3 mM; under these conditions, DNA was hydrolyzed practically entirely to 120-140 bp oligomers. With an increase in SAM concentration, the WEN2 activity decreases. During hydrolysis of methylated DNA, the dependence of the enzyme activity on SAM concentration is similar, but the degree of DNA hydrolysis was significantly lower than that of unmethylated DNA. Thus, it again demonstrates that, unlike WEN1 [8], the endonuclease WEN2 preferentially hydrolyzes unmethylated DNA.

We have observed earlier that SAH and SiBA, the structural SAM analogs devoid of active methyl group and the competitive inhibitors of DNA methylation, are allosteric effectors stimulating like SAM the DNA hydrolysis with endonuclease WEN1 [8]. Contrary to strong stimulating action on WEN1 [8], SAH and SiBA at concentration 0.3 mM stimulate hydrolysis of unmethylated phage DNA with WEN2 enzyme very weakly (Fig. 8). Nevertheless, the stimulation is visible, and these agents under some conditions similarly to SAM may modulate WEN2 activity. But SAM seems to be more specific activity modulator for WEN2 than for WEN1. This may indirectly indicate the possibly different structural organization of WEN2 and WEN1 endonucleases and, in particular, of their allosteric regulatory as well as reaction domains (centers).

So, we have isolated and characterized endonuclease WEN2 from vesicle fraction of cells of aging wheat coleoptiles. Similarly to endonuclease WEN1 [8], the WEN2 enzyme is a neutral Ca^{2+},Mg^{2+},Mn^{2+}-dependent endonuclease. Both enzymes are found in nuclei of apoptotic coleoptile cells. WEN2 preferentially hydrolyzes unmethylated double-stranded, but WEN1 methylated single-stranded λ phage DNA. Allosteric effectors such as SAM, SAH, and SiBA influence the activity of these enzymes in a different fashion. SAM strongly stimulates endonuclease WEN2 but only weakly the WEN1 activity. Competitive inhibitors of the DNA methylation reaction (SAM analogs SAH and SiBA) efficiently simulate endonuclease WEN1 [8] and weakly increase the activity of WEN2. These and other differences in the enzyme properties seem to be associated with different structure and organization of the endonucleases. We suggest that activity of these enzymes is induced and increased during apoptosis, and both these enzymes seem to participate in apoptotic DNA degradation and especially at the terminal stages of programmed cell death. It seems that each of these enzymes has its own place and time in different plant apoptotic events including caspase-dependent or caspase-independent apoptosis. It is important to learn whether the activity of these endonucleases can depend on caspases, like in some animal CAD (caspase-activated deoxyribonucleases) [14] or probably respective plant caspase-like proteases.

Thus, plants have a system of endonucleases that recognize DNA methylation status and are differently modulated with SAM, the universal methyl group donor. One of these enzymes (WEN2) prefers to hydrolyze double-stranded unmethylated DNA and is strongly activated with SAM, but the other (WEN1) preferably hydrolyzes single-stranded methylated DNA and is weakly modulated with SAM but much more with its analogs, SAH and SiBA. It seems that these enzymes should be functionally different as well.

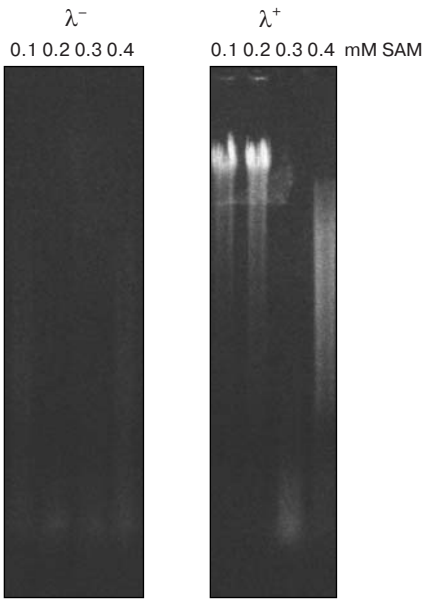

Fig. 7. Influence of SAM on WEN2 (0.25 μg) activity. Hydrolysis of unmethylated (λ^-) and methylated (λ^+) λ phage DNA.

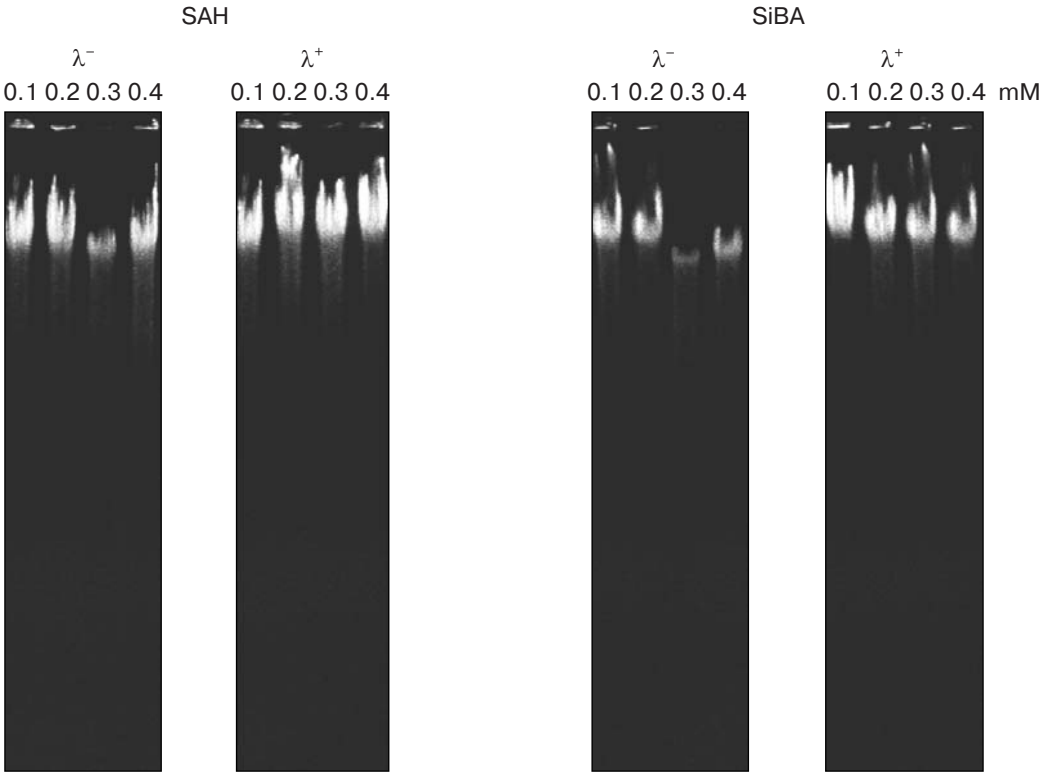

Fig. 8. Influence of SAH and SiBA on WEN2 (0.25 μg) activity. The numbers indicate concentration (mM) of SAM analogs in the reaction mixtures.

Detection of SAM-dependent plant endonucleases that are sensitive to methylation status of substrate DNAs may indicate the existence a restriction–modification (R–M) system in higher plants.

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