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CNG Site-Specific and Methyl-Sensitive Endonuclease WEN1 from Wheat Seedlings

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Abstract—Endonuclease WEN1 with apparent molecular mass about 27 kDa isolated from cytoplasmic vesicular fraction of aging coleoptiles of wheat seedlings has expressed site specificity action. This is a first detection and isolation of a site-specific endonuclease from higher eukaryotes, in general, and higher plants, in particular. The enzyme hydrolyzes deoxyribooligonucleotides of different composition on CNG (N is G, A, C, or T) sites by splitting the phosphodiester bond between C and N nucleotide residues in CNG sequence independent from neighbor nucleotide context except for CCCG. WEN1 prefers to hydrolyze methylated λ phage DNA and double-stranded deoxyribooligonucleotides containing 5-methylcytosine sites (m^5CAG, m^5CTG) compared with unmethylated substrates. The enzyme is also able to hydrolyze single-stranded substrates, but in this case it splits unmethylated substrates predominantly. Detection in wheat seedlings of WEN1 endonuclease that is site specific, sensitive to the substrate methylation status, and modulated with S-adenosyl-*L*-methionine indicates that in higher plants restriction–modification systems or some of their elements, at least, may exist.

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In eukaryotes the endonucleases play an important role in many genetic processes: these enzymes take part in DNA replication, recombination, repair, and DNA degradation particularly at the terminal stages of apoptosis [1].

There are at least two classes of eukaryotic endonucleases in plants – Ca^{2+},Mg^{2+}-dependent and Zn^{2+}-dependent endonucleases [1]. Many endonucleases different in properties and activities have been detected in wheat plants; it was established that the endonuclease set in plants is tissue specific, different in the nucleus and mitochondria, and changes significantly with age [2, 3]. Many plant endonucleases are strongly activated during plant aging and under influence of various stress factors including H_2O_2 [1]. Unfortunately, the site specificity of the action of plant endonucleases is practically unknown. There are only some data indicating that plant endonucleases, unlike known bacterial restriction endonucleases, are devoid of expressed site-specific action [1].

We have isolated two Ca^{2+},Mg^{2+}-dependent endonucleases (WEN1, WEN2) from aging wheat coleoptiles and studied some of their properties [4, 5]. The unknown earlier, unusual property of these eukaryotic enzymes is their ability to discriminate between substrate DNA different in methylation status. Besides, it is shown for the first time that the action of these plant endonucleases is modulated with S-adenosyl-*L*-methionine (SAM, AdoMet) like it is in some prokaryotic restriction endonucleases [4, 5], and with histone H1 [6].

This work describes the results of the investigation of the site specificity action of endonuclease WEN1 isolated from aging wheat coleoptiles.

MATERIALS AND METHODS

Isolation of cytoplasmic vesicle fraction from wheat coleoptiles. Seeds of winter wheat (Mironovskaya 808 variety) were germinated in darkness in a thermostat at 26°C, and then seedlings were grown under the same conditions for 8 days. The seedlings were thoroughly washed with tap water, and the coleoptiles were separated. At this

Abbreviations: BSA, bovine serum albumin; FAM, 5-carboxyfluorescein; SAM (AdoMet), S-adenosyl-*L*-methionine; WEN, wheat endonuclease.

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plant growth stage under the conditions described an intense apoptosis takes place in coleoptiles [7]. Isolated coleoptiles (1 g) were homogenized at 4°C in 5 ml homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.4 M sucrose, 1 mg/ml bovine serum albumin, BSA). Nuclei were isolated from the homogenate by 15 min centrifugation at 600*g*. A vesicle fraction was obtained from the supernatant by 15 min centrifugation at 3000*g*. The pellet of the vesicular fraction was washed with homogenization buffer without BSA.

Isolation and purification of endonuclease WEN1. 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. The protein with endonuclease activity was isolated from the extract and purified by ion-exchange chromatography on DEAE-cellulose in NaCl concentration gradient (0-0.5 M) and subsequent gel filtrations trough Superdex 200, Superdex 75, and Toyopearl HW-50 in 10 mM Tris-HCl buffer, pH 7.5, as described earlier [4].

The protein concentration was determined spectrophotometrically by the absorbance measurement at $\lambda = 280$ nm as well as according to Bradford's method [8].

Molecular mass of proteins was determined by electrophoresis in 12.5% SDS-polyacrylamide gel according to Laemmli's procedure [9]. The markers used were phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carboanhydrase (30 kDa), soya trypsin inhibitor (20 kDa), and lactalbumin (14.4 kDa).

The detection and visualization of the endonuclease activities were performed directly by protein electrophoresis in 12.5% polyacrylamide gel containing 0.2 mg/ml calf thymus DNA. This commercial DNA was preliminary dissolved twice in bidistilled water and precipitated with ethanol (70%). After electrophoresis the gel was washed free of SDS with stirring in distilled water for 2 h and water changing after each 15 min (first gel washing was done with 0.5% Triton X-100). After washings the gel was placed in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, and incubated overnight at 37°C. Then ethidium bromide was added (up to 0.0025%) to buffer with gel, and after 15 min incubation the areas of DNA hydrolysis in gel were visualized in UV-light.

Determination of endonuclease activity. On determination of endonuclease activity the total wheat DNA, unmethylated λ phage (dcm^-, dam^-) DNA, and methylated λ phage (dcm^+, dam^+) DNA were used as substrates. The phage DNAs were obtained from the same phage particles but grown up in the *E. coli* host strain (*dam*, *dcm*)$^-$ or (*dam*, *dcm*)$^+$ cells. Unlike unmethylated phage DNA (*dam*, *dcm*)$^-$, the methylated phage DNA (*dam*, *dcm*)$^+$ contained 5-methylcytosine residues in Cm^5CWGG sequences and N^6-methyladenine residues 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 DNA, and total volume of reaction mixture was adjusted up to 10 μl with bidistilled water. The reaction mixture was incubated at 37°C for 2 h (if not specially indicated), then the application buffer (2 μl) containing 10% glycerol, 0.01% bromophenol blue, and xylene cyanol in Tris-borate buffer (TBE) was added, and the mixture was applied on the 0.9% agarose gel in TBE buffer with 0.0005% ethidium bromide and electrophoresed. After electrophoresis the products of DNA hydrolysis were visualized in UV-light.

Hydrolysis of oligonucleotides and FAM-oligonucleotides with endonuclease WEN1 and analysis of hydrolysis products by electrophoresis in 20% polyacrylamide gel. Deoxyribooligonucleotides and their fluorescence-labeled with 5-carboxyfluorescein (FAM) derivatives were synthesized and kindly presented to us by Syntol (Russia). Synthetic deoxyribooligonucleotides (1 μg) were hydrolyzed with endonuclease WEN1 ($2 \cdot 10^{-8}$ M) in the presence of 3 mM $MgCl_2$ for 2 h at 37°C. The hydrolysis products were separated by electrophoresis in 20% polyacrylamide gel in Tris-borate buffer containing 0.05 M EDTA. The gels were immersed in solution of ethidium bromide for 2-3 min and analyzed then in a Fujifilm FLA 3000 photoimager (Japan). Quantitative analysis of fluorescence-labeled oligonucleotides was carried out in the same apparatus at 413-nm wavelength without preliminary gel treatment with ethidium bromide.

Chromatographic separation of oligonucleotides was performed on a C-18 column in the acetonitrile linear concentration gradient (0-60%) in a Biologic DuoFlow chromatograph (BioRad, USA).

RESULTS AND DISCUSSION

As described earlier [4], the cytoplasmic Ca^{2+},Mg^{2+}-dependent wheat endonuclease WEN1 with apparent molecular mass about 27 kDa was isolated from vesicle fraction of aging wheat coleoptiles. The enzyme is able to form dimers (Fig. 1) and seems to be more active in a dimeric form.

WEN1 is sensitive to DNA methylation status, the enzyme activity is modulated with S-adenosyl-*L*-methionine, and it prefers to hydrolyze methylated denatured (single-stranded) λ phage DNA (Fig. 2) compared with analogous undenatured unmethylated DNA. Unfortunately, on analysis of products of hydrolysis of λ phage DNA with endonuclease WEN1 discrete clear-cut bands (fragments) in agarose gel were not observed (Fig. 2). This may be due, in particular, to the presence of very many relatively short recognition sites in DNA that are split with endonuclease WEN1. In the presence of Mg^{2+} (3 mM) in the reaction mixture the phage DNA under conditions described are hydrolyzed completely to oligonucleotides of definite molecular mass with lengths of about 120-140 bp (Fig. 2a, lane *3*). Hydrolysis of unmethylated and methylated phage DNAs with endonuclease WEN1 at

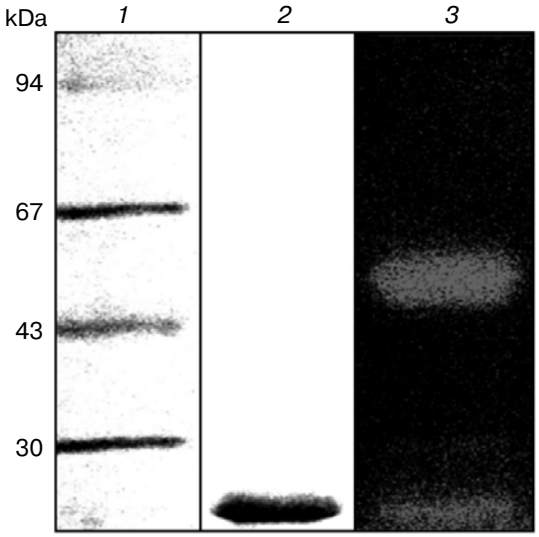

Fig. 1. Electrophoregrams of proteins in 12.5% polyacrylamide gel: *1*) protein markers: carboanhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), phosphorylase (94 kDa); *2*) denatured WEN1; *3*) activity of undenatured WEN1 enzyme in gel.

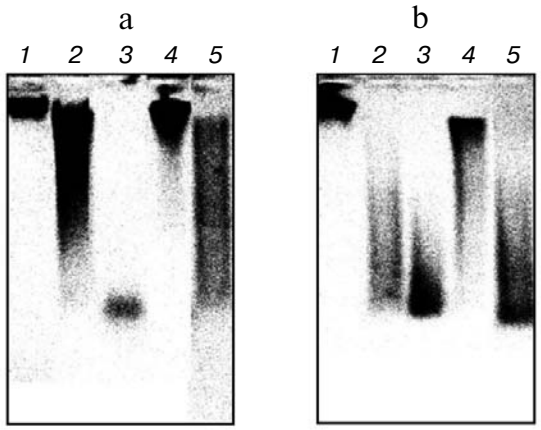

Fig. 2. Electrophoregrams of λ phage DNA and their hydrolysis products in 1.2% agarose: a) unmethylated DNA; b) methylated DNA. *1*) DNA (1 μg); *2*) lane *1* + WEN1 (3 μl, $2 \cdot 10^{-8}$ M); *3*) lane *2* + Mg^{2+} (3 μM); *4*) lane *2* + SAM (1 μM); *5*) DNA + WEN1 (3 μl, $2 \cdot 10^{-8}$ M), temperature of reaction mixture is 65°C.

65°C is much more intense compared with that at 37°C (Fig. 2, a and b, lanes *5*).

Contrary to the quite general opinion on the unspecific nature of DNA hydrolysis with higher plant endonucleases, the character of λ phage DNA hydrolysis observed, in general, may indicate that wheat endonuclease WEN1 has some site-specific action. In principle, the same conclusion may be drawn also from our own results that showed that endonuclease WEN1, contrary to DNA hydrolysis, does not hydrolyze monotonous polymers such as polydeoxyribo-A, polydeoxyribo-T, polydeoxyribo-C, polydeoxyribo-AT, polydeoxyribo-CI of various length (containing 20 and more nucleotide residues) (Table 1). It appeared to be clear that for hydrolysis of oligonucleotides or DNA with WEN1 the enzyme needs to operate with some alternative specific sequences (sites) (Table 2). To learn more about it, different in primary structure oligonucleotides (including fluorescence-labeled ones) were synthesized by Syntol according to our design. Single incorporations of some nucleotide combinations into monotonous structures (GC in polydeoxyribo-A or CG in polydeoxyribo-T) did not make such oligonucleotides available for hydrolysis with endonuclease WEN1 (Table 1). Insertion of GAT fragment into polydeoxyribo-C with formation in a chain of GATC site recognized by dam endonuclease or incorporation of GATA or GATG sites in this oligonucleotide do not lead to formation of a substrates sensitive to hydrolysis with the enzyme. This allowed us, in particular, to conclude that WEN1, at least, does not possess dam endonuclease site specificity. The enzyme did not hydrolyze oligonucleotides containing only CG sequences (Table 1) that are known target sites for DNA methylation in eukaryotes [10]. Another known methylation site in eukaryotic DNA is the CNG sequence; in higher plant DNA more than 30% of 5-methylcytosine residues are located in these particular sequences [10].

We decided to investigate the action of the endonuclease WEN1 on different deoxyribooligonucleotides containing CNG sequences. It is worth noting that all deoxyribooligonucleotides used containing CNG sites in any nearest neighboring nucleotide context, except for CCCG, were hydrolyzed with WEN1 enzyme (Table 2). The enzyme did not hydrolyze the deoxyribooligonucleotides with CNC sites.

Oligonucleotide G CGG CGG ATA CGG CGG ATG CGG CG with a fluorescent label (FAM) at the 3′-

Table 1. Deoxyribooligonucleotides (5′→3′) resistant to endonuclease WEN1

1	Poly(dA)
2	Poly (dAT)
3	Poly(dCI)
4	dAAA AAA AAA AAA AAA AAA AAA AAA
5	dCCC CCC CCC CCC CCC CCC CCC CCC
6	dAAA AAA AAA AGC AAA AAA AAA AAA AAA
7	dTTT TTT TTT TCG TTT TTT TTT TTT TTT
8	dCGC GCG CGC GCG CGC GCG CGC GCG
9	dCCC CCC GAT CCC CCC GAT CCC CCC
10	dCCC GAT ACC CCC CCC GAT ACC CCC
11	dCCC GAT GCC CCC CCC GAT GCC CCC
12	dCGC GCG CGC CCC GCG CGC GCG CGC

Table 2. Deoxyribooligonucleotides (5′→3′) hydrolyzed with endonuclease WEN1

1	GCG GCG GAT ACG GCG GAT GCG GCG(FAM)
2	(FAM)GCG GCG GAT ACG GCG GAT GCG GCG
3	GCG GCG GA(T-FAM) ACG GCG GAT GCG GCG
4	(FAM) CGC CGC CAG GCG CCG CCG CG
5	CGC CGC CAG GCG CCG CCG CG(FAM)
6	(FAM)GCG GCG GCG CTG GGC GGC GC
7	GCG GCG GCG CTG GGC GGC GC(FAM)
8	GCG GCG GCG C(T-FAM)G GGC GGC GC
9	CGC GCG CGC GCG GCG CGC GCG CGC
10	CGC GCG CGC GCA GCG CGC GCG CGC
11	CGC GCG CGC GCC GCG CGC GCG CGC
12	CGC GCG CGC GCT GCG CGC GCG CGC
13	GCG GCG CCA GGG CGG CGG CG
14	GCG GCG C(m^5)CA GGG CGG CGG CG
15	CGC CGC CGC CCT GGC GCC GC
16	CGC CGC CGC C(m^5)CT GGC GCC GC

end containing five CNG (CGG) sites was hydrolyzed with WEN1 enzyme with formation of at least six fragments, which corresponds to the CNG site number in the initial oligonucleotide (Fig. 3, lane *2*). The number of fragments formed on hydrolysis of the same oligonucleotide but with fluorescein label at the 5′-end is markedly less (Fig. 3, lane *4*) compared with that of 5′-end labeled oligonucleotide. Similar product number was observed also on hydrolysis of oligonucleotide containing fluorescein in the middle of the substrate (not shown). This means that FAM in the middle or at the 5′-end of the substrate prevents partially the hydrolysis of the oligonucleotide with endonuclease WEN1. Besides, it shows once more that WEN1 is, in fact, an endonuclease. It seems that the enzyme hydrolyzes initially the oligonucleotide in the 5′-end region.

Two fragments similar in length (represented as a single band on electrophoregram) originate on WEN1 hydrolysis of various oligonucleotides containing single CNG site (CGC GCG CGC G**CG GC**G CGC GCG CGC, CGC GCG CGC G**CA** GCG CGC GCG CGC, CGC GCG CGC G**CT GC**G CGC GCG CGC and CGC GCG CGC G**CC GC**G CGC GCG CGC) (Fig. 4). In control these oligonucleotides are represented by two components (Fig. 4, lanes *1*, *3*, *5*, *7*), one of which is double-stranded (a) and the other single-stranded (b). Under enzyme action the double-stranded structures in these oligonucleotides with CGG, CTG, and CCG sites (lanes *2*, *6*, and *8*, respectively) but not with CAG site (lane *4*) disappear completely. Thus, in principle, the enzyme is able to split both double-stranded and single-stranded oligonucleotides.

To establish where the enzyme splits the phosphodiester bond in the CNG site, the relatively long deoxyribooligonucleotide consisting of 90 nucleotide residues with single CNG (CCG) target site was used for enzymatic hydrolysis (AAA GGG CCC TTT GGG AAA CCC TTT GGG **CCG** TTT AAA GGG CCC TTT AAA GGG TTT CCC AAA GGG TTT CCC AAA GGG TTT CCC AAA GGG TTT). Two hydrolysis products such as

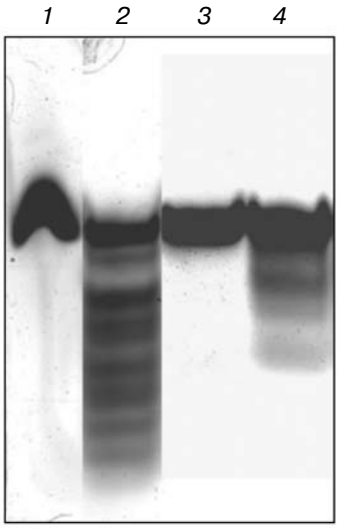

Fig. 3. Electrophoregrams of fluorescence-labeled deoxyribooligonucleotides and their hydrolysis products in 20% polyacrylamide gel: *1*) (5′) GCG GCG GAT ACG GCG GAT GCG GCG(FAM) (3′); *2*) lane *1* + endonuclease WEN1; *3*) (5′) (FAM)GCG GCG GAT ACG GCG GAT GCG GCG (3′); *4*) lane *3* + endonuclease WEN1. FAM stands for fluorescein.

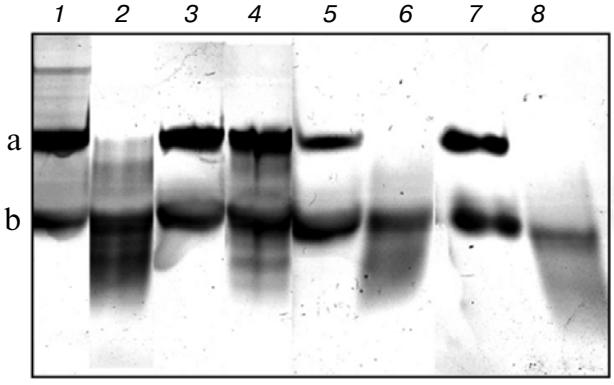

Fig. 4. Electrophoregrams (in 20% polyacrylamide gel) of deoxyribooligonucleotides containing a single CNG site and of products of their hydrolysis with endonuclease WEN1: *1*) CGC GCG CGC G**CG GC**G CGC GCG CGC; *2*) hydrolysis products of *1*; *3*) CGC GCG CGC G**CA GC**G CGC GCG CGC; *4*) hydrolysis products of *3*; *5*) CGC GCG CGC G**CT GC**G CGC GCG CGC; *6*) hydrolysis products of *5*; *7*) CGC GCG CGC G**CC GC**G CGC GCG CGC; *8*) hydrolysis products of *7*. a) Double-stranded; b) single-stranded deoxyribooligonucleotides.

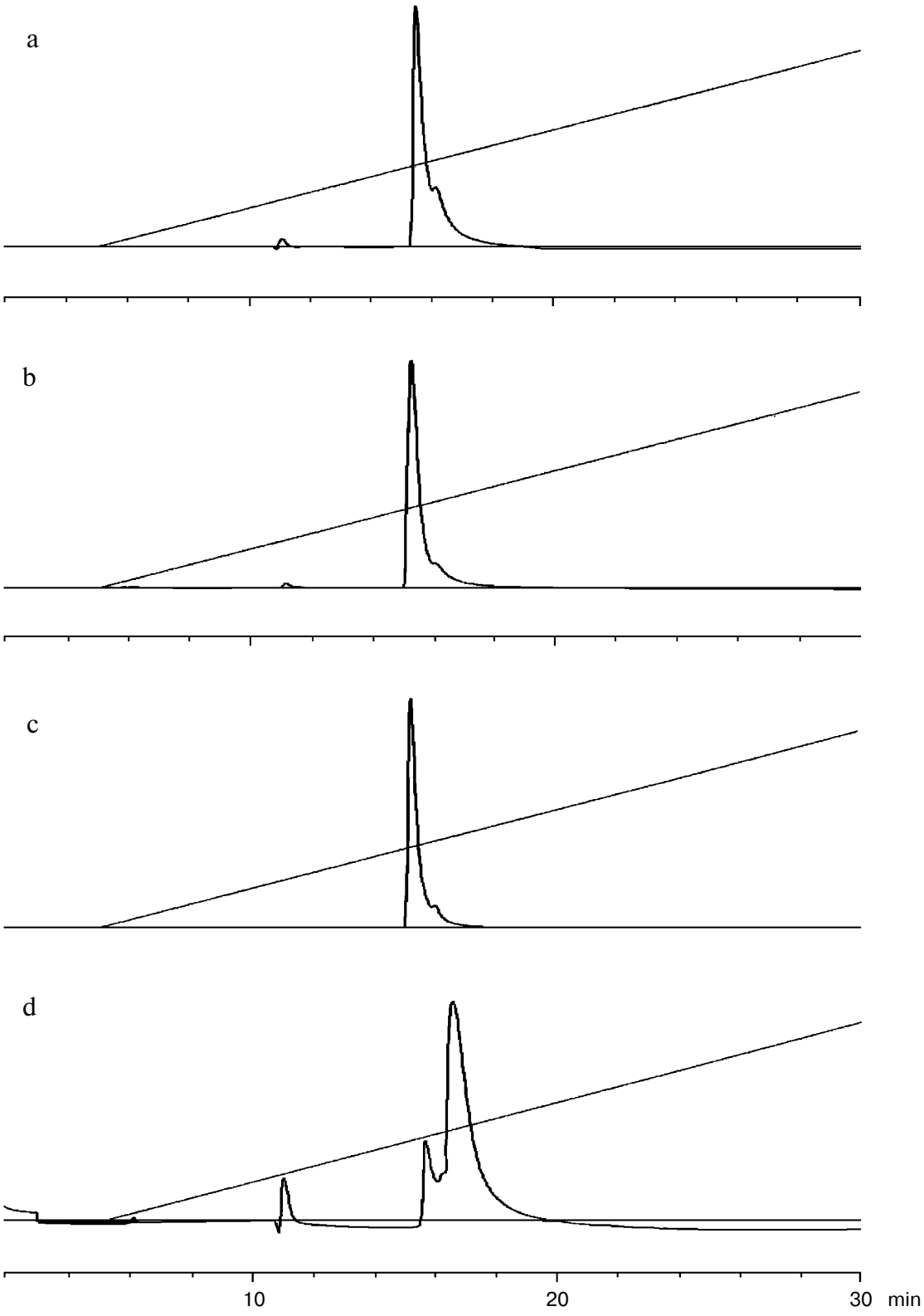

Fig. 5. Highly efficient liquid chromatography of deoxyribooligonucleotides on a C18 column. a) AAA GGG CCC TTT GGG AAA CCC TTT GGG **CC**; b) AAA GGG CCC TTT GGG AAA CCC TTT GGG **C**; c) AAA GGG CCC TTT GGG AAA CCC TTT GGG; d) products of hydrolysis of AAA GGG CCC TTT GGG AAA CCC TTT GGG **CCG** TTT AAA GGG CCC TTT AAA GGG TTT CCC AAA GGG TTT CCC AAA GGG TTT CCC AAA GGG TTT with endonuclease WEN1. On abscissa the time of chromatography (min) is shown.

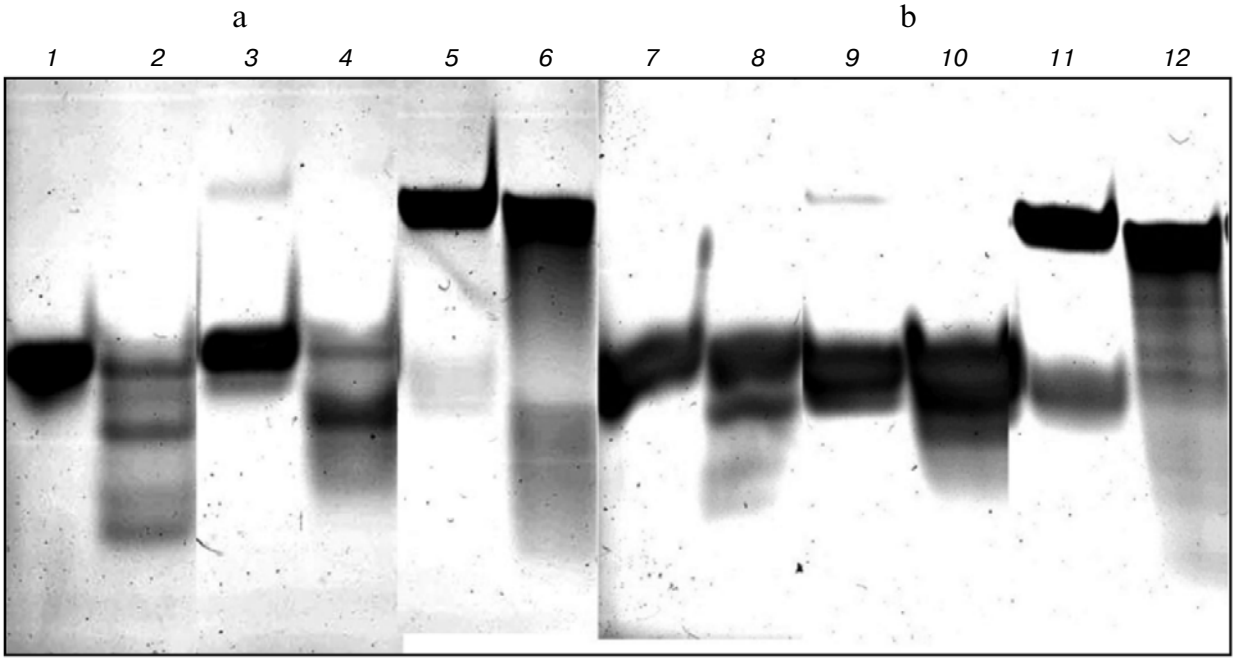

Fig. 6. Electrophoregrams (in 20% polyacrylamide gel) of unmethylated (a) and methylated (b) deoxyribooligonucleotides and products of their hydrolysis with endonuclease WEN1. *1*) GCG GCG CCA GGG CGG CGG CG; *2*) hydrolysis products of *1*; *3*) CG CGC CGC CCT GGC GCC GC; *4*) hydrolysis products of *3*; *5*) lanes *1* + *3* annealed; *6*) hydrolysis products of *5*; *7*) GCG GCG C(m^5)CA GGG CGG CGG CG; *8*) hydrolysis products of *7*; *9*) CG CGC CGC C(m^5)CT GGC GCC GC; *10*) hydrolysis products of *9*; *11*) lanes *7* + *9* annealed; *12*) hydrolysis products of *11*.

AAA GGG CCC TTT GGG AAA CCC TTT GGG **C** and **CG** TTT AAA GGG CCC TTT AAA GGG TTT CCC AAA GGG TTT CCC AAA GGG TTT CCC AAA GGG TTT were detected by highly efficient chromatography on a C18 column in a linear concentration gradient of acetonitrile. Judging by the time of elution from the column, one of these products was compared with specially synthesized oligonucleotides AAA GGG CCC TTT GGG AAA CCC TTT GGG (c), AAA GGG CCC TTT GGG AAA CCC TTT GGG **C** (b), and AAA GGG CCC TTT GGG AAA CCC TTT GGG **CC** (a) (Fig. 5) and identified as oligonucleotide (b). Thus, endonuclease WEN1 hydrolyzes a deoxyribooligonucleotide in the CNG site between the C and N residues (where N is any DNA nucleotide).

Deoxyribooligonucleotide GCG GCG CCA GGG CGG CGG CG containing four CNG sites was split by the enzyme to at least four fragments (Fig. 6, lane *2*), but its methylated analog containing 5-methylcytosine in the m^5CAG sequence was hydrolyzed only to two fragments (Fig. 6, lane *8*). This means that a methyl group in the 5-methylcytosine residue prevents full hydrolysis of this oligonucleotide with the WEN1 enzyme.

Double-stranded unmethylated oligonucleotide obtained by annealing of complementary GCG GCG CCA GGG CGG CGG CG and CGC CGC CCT GGC GCC GC was hydrolyzed by the enzyme weakly (Fig. 6, lane *6*), but its methylated double-stranded analog containing m^5CAG in one chain and GTm^5C in the opposite one was hydrolyzed with formation of at least four relatively long fragments (Fig. 6, lane *12*). Thus, methylated double-stranded structures containing m^5CAG and m^5CTG were hydrolyzed much better compared with unmethylated ones. This corresponds to our data (Fig. 2 and [4, 5]) that WEN1 enzyme prefers to hydrolyze methylated DNA compared with unmethylated DNA. The enzyme is able to hydrolyze also the single-stranded oligonucleotides, but in this case it prefers to deal with unmethylated substrates (Fig. 6).

Thus, we described here the detection, isolation, and partial characterization of the first higher eukaryotic endonuclease with expressed site-specific action. The enzyme hydrolyzes deoxyribooligonucleotides at CNG sites by splitting the phosphodiester bond between the C and N residues (N = G, A, T, C) in any neighboring nucleotide context except for CCCG.

It cannot be ruled out that the action of this enzyme is somehow associated or even coordinated with the action of DNA methyltransferase CMT3 that methylates cytosine residue in CNG sequences. This particular DNA methylation plays an essential role in chromatin organization and specific regulation of expression of many genes in plants and animals in various stress situations [11]. The action of endonuclease WEN1 under some conditions can be suppressed with S-adenosyl-*L*-methionine (SAM); therefore, it is quite logical to assume that under

the influence of SAM in conditions most favorable for DNA methylation the activity of WEN1 can be decreased, which is important for safety of DNA structures newly methylated with DNA methyltransferase CMT3. This situation seems to be quite probable in a plant cell.

We have already mentioned that enzyme WEN1 was isolated from the fraction of wheat coleoptile cytoplasmic vesicles containing mitochondria. It seems that this enzyme has mitochondrial origin. Analogous endonuclease activity with similar molecular mass was found also in nuclei isolated from wheat coleoptiles [5]. It can be suggested that the enzyme is encoded in the nucleus and after transcription of the corresponding gene and protein formation, WEN1 is transported into mitochondria. Activity of WEN1 in coleoptiles increases with age and is maximal in senescent coleoptiles [5].

The abilities of the enzyme to discriminate between methylated and unmethylated DNAs, recognize the methylation status of double-stranded deoxyribooligonucleotides and definite specific sites (CNG), as well as to be modulated with SAM [4, 5] make it to some extent similar in these properties with prokaryotic restriction endonucleases. Taking into consideration the probable bacterial origin of mitochondria in eukaryotes, it is quite reasonable to suggest that bacteria, in particular, could bring with them such restriction endonucleases into the eukaryotic cell, and the genes coding them could appear somehow in the nucleus.

Anyway, the detection of the unique endonuclease WEN1 in wheat seedlings shows that higher plants may have a restriction–modification (R-M) system or at least some its elements. From our point of view these epigenetic systems may take part in various important events of plant life including selective deletion of genetic material, genome instability, and apomixis.

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