Wheat (*Triticum vulgare*) Chloroplast Nuclease ChSI Exhibits 5′ Flap Structure-Specific Endonuclease Activity†

Anna Przykorska,*,‡ Karina Solecka,‡ Krzysztof Olszak,‡ Gerard Keith,§ Barbara Nawrot,|| and Elzbieta Kuligowska‡

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland, Institut de Biologie Moléculaire et Cellulaire, CNRS, UPR 9002, 67084 Strasbourg, France, and Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Sienkiewicza 112, 90-363 Lodz, Poland

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ABSTRACT: The structure-specific ChSI nuclease from wheat (*Triticum vulgare*) chloroplast stroma has been previously purified and characterized in our laboratory. It is a single-strand-specific DNA and RNA endonuclease. Although the enzyme has been initially characterized and used as a structural probe, its biological function is still unknown. Localization of the ChSI enzyme inside chloroplasts, possessing their own DNA that is generally highly exposed to UV light and often affected by numerous redox reactions and electron transfer processes, might suggest, however, that this enzyme could be involved in DNA repair. The repair of some types of DNA damage has been shown to proceed through branched DNA intermediates which are substrates for the structure-specific DNA endonucleases. Thus we tested the substrate specificity of ChSI endonuclease toward various branched DNAs containing 5′ flap, 5′ pseudoflap, 3′ pseudoflap, or single-stranded bulged structural motifs. It appears that ChSI has a high 5′ flap structure-specific endonucleolytic activity. The catalytic efficiency (k_{cat}/K_M) of the enzyme is significantly higher for the 5′ flap substrate than for single-stranded DNA. The ChSI 5′ flap activity was inhibited by high concentrations of Mg^{2+}, Mn^{2+}, Zn^{2+}, or Ca^{2+}. However, low concentrations of divalent cations could restore the loss of ChSI activity as a consequence of EDTA pretreatment. In contrast to other known 5′ flap nucleases, the chloroplast enzyme ChSI does not possess any 5′→3′ exonuclease activity on double-stranded DNA. Therefore, we conclude that ChSI is a 5′ flap structure-specific endonuclease with nucleolytic activity toward single-stranded substrates.

The integrity of genomes of living organisms depends on several processes such as DNA replication, recombination, and repair. During DNA metabolism, intermediate structures of branched duplexes containing a free single-stranded 5′ end, called 5′ flap DNAs, are often formed (*1*−*7*). These intermediates are recognized by specific 5′ flap nucleases, and a single-stranded part of 5′ flap DNA is removed (*8*).

Among 5′ flap nucleases, mammalian flap endonuclease-1 (FEN-1)[1] hydrolyzes 5′ flap DNA endonucleolytically, independently of flap length and regardless of its primary sequence. In addition, this enzyme possesses 5′→3′ double-strand-specific exonuclease activity (*9*). It has also been shown that FEN-1 is able to cleave the single strands in double 5′ and 3′ flap branched DNAs (*10*), which have been proposed to arise in double-strand break repair (*7*, *9*), and in homologous recombination (*6*). FEN-1 has also been shown to be implicated in the removal of the initiator RNA in the processing of Okazaki fragments during replication (*11*). In addition, FEN-1 appears to be involved in nucleotide excision repair, this being the major pathway to remove damaged nucleotides from DNA (*4*).

The mammalian FEN-1 gene has been cloned and its primary sequence compared to sequences of other structure-specific nuclease genes. It was found that the FEN-1 gene is highly homologous with the *Saccharomyces cerevisiae* genes coding the RTH1 (also known as RAD27) and RAD2 nucleases (*4*, *11*). Mutations in the *S. cerevisiae* RTH1 gene result in increased sensitivity to alkylating agents and ultraviolet radiation or lead to an increase in the rate of instability of simple repetitive sequences in the DNA by as much as 280-fold (*11*). It has been suggested that mutations in the human FEN-1 could cause colorectal and other cancers that are known to result from sequence duplications (*12*, *13*). In addition, RTH1 has been shown to be implicated in preventing trinucleotide repeat (TNR) expansion and contraction. Deletion mutants in the gene encoding this protein result in length-dependent destabilization of CTG tracts and in their expansion frequency, suggesting that defects in the human counterpart of RTH1 could lead to genetic diseases such as myotonic dystrophy, Huntington disease, several

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* To whom correspondence should be addressed. Phone: +48 22 5922414. Fax: +48 39121623. E-mail: annap@ibb.waw.pl.

‡ Institute of Biochemistry and Biophysics, Polish Academy of Sciences.

§ Institut de Biologie Moléculaire et Cellulaire, CNRS.

|| Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences.

[1] Abbreviations: 3′-UTR, 3′-untranslated region; ChSI, nuclease from wheat chloroplasts; EDTA, ethylenediaminetetraacetic acid; FEN-1, flap endonuclease-1; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS, sodium dodecyl sulfate; TBE, Tris−borate−EDTA buffer; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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ataxias, and fragile X syndrome, which are relevant to TNR expansion (*14*, *15*).

Enzymes homologous with FEN-1 have also been found in viruses, archaebacteria, and eubacteria as well as in higher plants. However, the known viral members of 5′ nucleases, the T5 5′→3′ exonuclease (*16*) and the T4 RNase H (*17*), show relatively low sequence similarity with FEN-1 (*18*). In contrast, the archaebacterial enzymes (*19*, *20*) from *Archaeglobus fulgidus*, *Methanoccocus jannaschii*, and *Pyrococcus furiosus* share approximately 75% similarity with FEN-1, in two well-conserved domains, the N-terminal and I-intermediate domains (*21*, *22*). The eubacterial enzymes share homology with FEN-1 in the N-terminal domain (*8*) and act either as polymerases, 5′→ 3′ exonucleases, or flap endonucleases (*23*). These enzymes are the *Escherichia coli* DNA polymerase 1 and *Thermus aquaticus* DNA polymerase (Taq polymerase) that participate in removal of RNA primers in maturation of the lagging strand during replication, as well as in repair of damaged nucleotides (*24*, *25*). In contrast, all known eukaryotic DNA polymerases lack this flap activity.

In the case of plants, two 5′ flap-specific nucleases have been described to date. One of them was purified from cauliflower (*Brassica oleracea*) influorescence. This enzyme has weak 5′→3′ double-strand-specific exonuclease activity and cleaves endonucleolytically at the 3′ end of the unpaired region in stem−loop structures (*26*). Another plant FEN-1, the rice counterpart (*Oryza sativa*) OsFEN-1, has been cloned and overexpressed (*27*). It was shown that OsFEN-1 possesses both 5′ flap endonuclease and 5′→3′ double-stranded DNA exonuclease activities. OsFEN-1 transcripts are strongly expressed in proliferating tissues, and expression of the enzyme may be required for cell growth and organ formation. Comparison of amino acid sequences of OsFEN-1 with several eukaryotic 5′ nucleases shows that the enzyme shares 53% sequence identity with human FEN-1, 56% with *Xenopus laevis* FEN-1, 50% with *S. cerevisiae* RAD27, and 52% with *Saccharomyces pombe* Rad2. This suggests that OsFEN-1 is the plant counterpart of FEN-1 (*27*).

We have previously purified and characterized the structure-specific ChSI nuclease from wheat (*Triticum vulgare*) chloroplast stroma. This enzyme is a single-strand-specific endonuclease that catalyzes the hydrolysis of either DNA or RNA to acid-soluble 5′-phosphate-terminated products. In addition, ChSI converts the supercoiled covalently closed plasmid pBR322 DNA into the open circular form and subsequently into the linear form (*28*). This enzyme was characterized as a very sensitive and useful tool for secondary and tertiary structure investigations of RNAs because it (i) does not require the addition of divalent cations for stability and activity, (ii) can be used in the presence of such ions, (iii) cleaves single-stranded or relaxed regions in either DNA or RNA and can even recognize single bulged-out nucleotides, and (iv) is very stable and can be kept frozen without loss of activity for several years. Nuclease ChSI was used to study the secondary structure of various molecules of RNA, including native bovine and *X. laevis* $tRNAs^{Sec}$ with large variable arms (*29*), the 3′ untranslated region (3′ UTR) of vimentin mRNA (*30*), and tRNA complexed with tRNA (guanosine-1) methyltransferase (*31*).

Although the ChSI enzyme has been initially characterized and used as a structural probe, its biological function is still unknown. However, taking into consideration the localization of the ChSI enzyme inside chloroplasts, which possess their own DNA that is generally highly exposed to UV light and often affected by numerous redox reactions and electron transfer processes (*32*), one could expect that this enzyme might be involved in DNA repair and therefore act as a 5′ flap nuclease. To gather more information about the possible function and the specificity of this plant nuclease, we have used several branched DNAs of the following structures: 5′ flap, 5′ pseudoflap, 3′ pseudoflap, and bulged DNAs that were subjected to the action of the ChSI nuclease.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (2500 Ci/mmol) was from ICN. T4 polynucleotide kinase (10 units/μL) was from Boehringer Mannheim.

Enzyme Purifications. Nuclease ChSI was purified from wheat (*T. vulgare*) chloroplast stroma according to the modified method as previously described (*28*). Chloroplasts were isolated from wheat leaves according to ref *33*. After subfractionation of the disrupted chloroplasts (*34*) the fraction containing the stroma was collected, dialyzed against 10 mM Tris-HCl buffer, pH 7.4 (buffer C), concentrated, and applied to a 2.5 × 20 cm column of DE-52 cellulose equilibrated with buffer C. Elution was performed with the same buffer, and 6 mL fractions were collected. The enzyme fractions were pooled, concentrated, applied to a 2 × 40 cm column of Sephadex G-75, equilibrated with buffer C, and eluted with the same buffer. The flow rate was 4.5 mL min^{-1}. The active nuclease fractions were pooled, concentrated, and rechromatographed on Sephadex G-75. All steps were carried out at 4 °C.

DNase activity was determined according to ref *35*. The reaction mixture contained, in a final volume of 1 mL, 75 μg of denatured DNA, 100 μL of enzyme solution, and 750 μL of 100 mM Tris-HCl buffer, pH 7.4. After 1 h of incubation at 37 °C the reaction was stopped by the addition of 3 mL of cold absolute ethanol, and the nondegraded substrate was removed by centrifugation at 14000*g* for 15 min. In the supernatant the absorbance was measured at 260 nm.

One unit of activity of the enzyme was defined as the amount of enzyme which, under the condition of assay, led to an increase of 0.1 in absorbance at 260 nm.

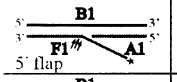
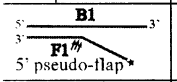
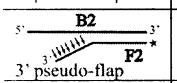
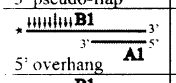
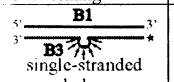
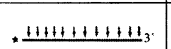
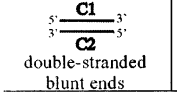
To detect DNase activity in a polyacrylamide gel, 0.3 mg/mL denatured DNA was added to the gel solution prior to polymerization. Proteins were separated on denatured DNA-containing SDS−polyacrylamide gels as described (*36*−*38*). SDS was then removed from the gel by soaking in 25% 2-propanol in 10 mM Tris-HCl, pH 7.4, and then in buffer alone. To digest the embedded denatured DNA, the gel was incubated for 14 h at 37 °C in 100 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$. The gel was then stained with 0.2% toluidine blue in 10 mM Tris-HCl, pH 7.4. Proteins with DNase activity appear as white bands. After being destained in 50% methanol and 12% acetic acid, the same gel was stained following a silver staining procedure (*38*). RnI nuclease was isolated from rye (*Secale cereale*) germ nuclei as described (*39*).

Oligonucleotides. The DNA oligonucleotides A1, B1, B2, C1, C2, and F1 (sequences as in Table 1) were purchased from the Department of Bioorganic Chemistry, Centre of

Table 1: Oligonucleotide Sequences

oligonucleotides	length (nt)	oligonucleotide sequence
A1	16	5′ CACGTTGACTACCGTC 3′
B1	30	5′ GGACTCTGCCTCAAGACGGTAGTCAACGTG 3′
B2	42	5′ CCAGTGAATTCGAGCTCGGTACCCGCTAGCGGGGATCCTCTA 3′
B3	35	5′ CACGTTGACTACCGTCAGTAGATGAGGCAGAGTCC 3′
D1	15	5′ GGACTGCTTGACATC 3′
C1	8	5′ GCAAAAGC 3′
C2	8	5′ GCTTTTGC 3′
F1	34	5′ GATGTCAAGCAGTCCTAACTTTGAGGCAGAGTCC 3′
F2	31	5′ TAGAGGATCCCCGCTAGCGGGCCAATCTTGA 3′

Table 2: Summary of ChSI Nuclease Activities on DNA Substrate

Structure	ChSI nuclease activity
 5′ flap	Structure-specific 5′ flap endonuclease
 5′ pseudo-flap	Structure-specific 5′ pseudo-flap endonuclease
 3′ pseudo-flap	Non-specific 3′ pseudo-flap endonuclease
 5′ overhang	Non- specific endonuclease
 single-stranded bulge	Non-specific endonuclease on single-stranded bulged out region
 single-stranded	Non-specific endonuclease
 double-stranded blunt ends	None

Molecular and Macromolecular Studies, Polish Academy of Sciences, Lodz, whereas B3, D1, and F2 were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Polish Academy of Sciences, Warsaw.

Oligonucleotide Constructs. The oligonucleotides were 5′ end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase in 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM dithiotheitol, and 1 mM spermidine buffer, pH 8.2. The reaction mixture was incubated for 30 min at 37 °C and stopped by adding buffer containing 7 M urea, 1 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue, pH 7.5. The labeled samples were purified by electrophoresis on a 15% polyacrylamide gel containing 8 M urea and TBE buffer (45 mM Tris base, 1.25 mM EDTA, and 45.3 mM boric acid, pH 8.3) at 200 V for 12 h.

The 5′-end-labeled oligonucleotides were further annealed to the corresponding unlabeled oligonucleotides in a 1:2 ratio (respectively radioactive and nonradioactive oligonucleotides) in 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl. All DNA substrates generated in this way are listed in Table 2.

Nuclease Assay. The 5′-^{32}P-labeled DNA substrates were incubated with ChSI nuclease in buffer A (20 mM Tris-HCl, pH 7.5) or with RnI in buffer B (10 mM acetate buffer, pH 5.4) at 37 °C for the indicated times. The reactions were stopped by the addition of stop solution (95% formamide, 10 mM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL xylene cyanol) and heating at 95 °C for 5 min. The samples were analyzed by 12% polyacrylamide−8 M urea gel electrophoresis and the results visualized by autoradiography or quantified using a PhosphorImager (Molecular Dynamics).

Melting Temperature Measurements. Samples for melting temperature measurements were prepared by hybridization of C1 [5′-d(GCAAAAGC)-3′] and C2 [5′-d(GCTTTTGC)-3′] oligomers at a concentration 4.2 μM of each oligomer in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 10 mM $MgCl_2$. Melting profiles were recorded after heating of the mixture to 70 °C followed by annealing to 5 °C with a temperature gradient of 0.5 °C/min. Oligonucleotides were kept for 5 min at 5 °C and then heated with a temperature gradient of 0.2 °C/min to 80 °C. The melting temperatures were calculated using the first-order derivative method. Measurements were carried out on a Cintra 40 instrument (GBC Australia).

Preparation of Double-Stranded DNA. Equal volumes of 10 μM solutions of oligonucleotides C1 and C2 were mixed, heated for 5 min at 95 °C, and cooled to room temperature over 1 h. A 10 μM stock solution of duplex C1/C2 was used for each digestion experiment.

Digestion of DNA with Nuclease ChSI. Water solutions of single-stranded oligodeoxyribonucleotide C1 or C2 or the 1:1 complex of C1 and C2 (1 μL of a 10 μM stock solution) were mixed with 1.5 μL of digestion buffer A and 5 μL (5 units) of enzyme solution. The mixture was kept on ice, and 1 μL samples were withdrawn after 30, 60, 120, and 240 min. Samples were placed on a sample plate, mixed with the matrix solution [1 μL of an 8/1 (v/v) mixture of 2,4,6-trihydroxyacetophenone (10 μg/mL in ethanol) and diammonium citrate (50 μg/mL in water)], and left to crystallize. MALDI-TOF mass spectra were collected on a Voyager-Elite instrument (PerSeptive Biosystems) in the reflector mode, at a resolution of 2000.

Digestion of DNA with Snake Venom Phosphorodiesterase (PDE I, 3′-Exonuclease). To a 1:1 complex of C1 and C2 (1 μL of a 10 μM stock solution) were added water (5.5 μL) and a solution of PDE I (1 μL of *Crotolus durissus* phosphorodiesterase, EC 3.1.15.1; Boehringer Mannheim GmbH, Germany; 3 milliunits/μL), and the mixture was incubated at 37 °C. One microliter samples were withdrawn from the reaction mixture after 10, 30, and 60 min, placed on the analysis plate, mixed with the matrix solution (1 μL), and left to crystallize. The MALDI-TOF mass spectra were then collected.

Digestion of DNA with Calf Spleen Phosphorodiesterase (PDE II, 5′-Exonuclease). A solution of a 1:1 complex of C1/C2 (1 μL of a 10 μM stock solution) was mixed with

water (5 μL) and 1 μL of PDE II (calf spleen phosphorodiesterase, EC 3.1.16.1; Sigma, St. Louis, MO; 1 μg/μL) and incubated at 37 °C. Samples of 1 μL were withdrawn from the reaction mixture after 30, 60, 120, and 240 min, placed on the sample plate, mixed with the matrix solution (1 μL), and left to crystallize. The MALDI-TOF mass spectra were then collected.

Determination of Kinetic Parameters. ChSI cleavage kinetics were performed under standard conditions, as described in the nuclease assay, using varying concentrations of DNA substrates, ranging from 0.5 to 5.0 nM, and a constant amount of ChSI enzyme (molecular mass = 29 kDa), 137 ng for 5′ flap, and 1073 ng for single-stranded DNA, in a final reaction volume of 4 μL. Reactions were incubated for 1 min at 37 °C and then stopped and heated at 95 °C for 5 min. The velocity was calculated by measuring product and substrate intensity on a gel using a PhosphorImager and ImageQuant version 5.2 software (Molecular Dynamics) and converted using the equation $v = \{I_1/(I_0 + 0.5I_1)t\}$[substrate], where t = time in seconds, I_1 = product intensity, and I_0 = final substrate intensity and the substrate concentration is expressed in nanomolar (21). This equation differs from the original one of Petruska et al. (*40*), who expressed the initial velocity of the reaction as a percent of product formed per unit time [$v = I_1(I_0 + 0.5I_1)/t$]. V_{max} and K_M values were calculated by directly fitting the data to the Michaelis−Menten equation using the Excell program. All reactions were repeated in triplicate.

Determination of Kinetic Parameters of Nuclease ChSI in the Presence of Magnesium Ions. Kinetic experiments were performed as above except that, at first, the ChSI nuclease was preincubated with 0.5 mM EDTA in buffer A for 15 min, then Mg^{2+} ions were added, and the enzyme was incubated for 15 min. The 5′ flap DNA substrate was then subjected to the action of ChSI for 2 min. The reaction was stopped, and the intensity of the products and substrate on the gel was used for determination of kinetic data, as described above.

RESULTS

Purity of the ChSI Preparation. ChSI nuclease was obtained from the stroma of wheat leaf chloroplasts by means of DE-52 cellulose and two-step Sephadex G-75 column chomatography as described in Materials and Methods. The final enzyme preparation was purified about 100 times in 19% yield and had a specific activity of 21500 units (mg of protein)$^{-1}$. The enzyme preparation stored at −20 °C was stable for several years.

The fractions of the highest activity collected from the last step of enzyme purification were concentrated and loaded onto a SDS−12% polyacrylamide gel containing denatured DNA. After electrophoresis and toluidine blue staining, a single band exhibiting nuclease activity, corresponding to a ca. 29 kDa protein, was detected (Figure 1A). After being destained followed by silver staining, the protein band with the same molecular mass replaced the nuclease activity band seen previously (Figure 1B).

ChSI Nuclease Has 5′ Flap Structure-Specific Activity. To test whether the ChSI nuclease has any specific 5′ flap endonuclease activity, we prepared a synthetic 5′ flap DNA construct containing oligonucleotides B1 and A1 and 5′-radiolabeled oligonucleotide F1 (as shown in Table 2). This DNA was treated with increasing amounts of nuclease ChSI. Reaction products were analyzed by 12% polyacrylamide sequencing gel electrophoresis (PAGE). As shown in Figure 2A, a primary event of the digestion after only 1 min of the reaction is the release of three major products. These are (i) the 22 nt oligomer, which is a product of a cut 2 nt proximal

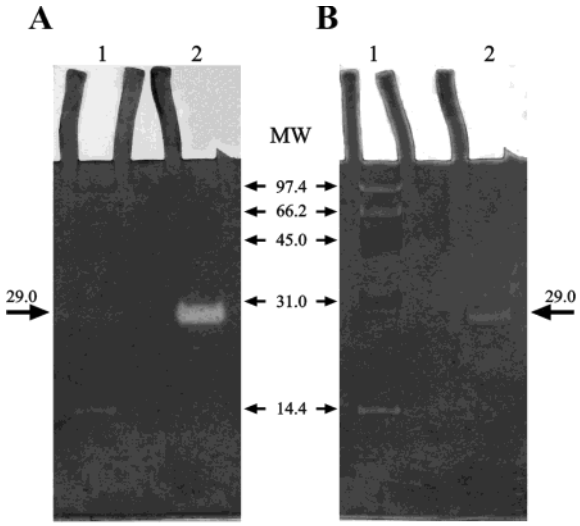


FIGURE 1: Analysis of ChSI purification by SDS−PAGE. Proteins were visualized by toluidine blue staining (A) and silver staining (B). Lanes: 1, protein molecular mass standards (kDa); 2, the fractions of the highest activity collected from the last step of enzyme purification. Arrows indicate the sizes of molecular mass standards and ChSI nuclease.

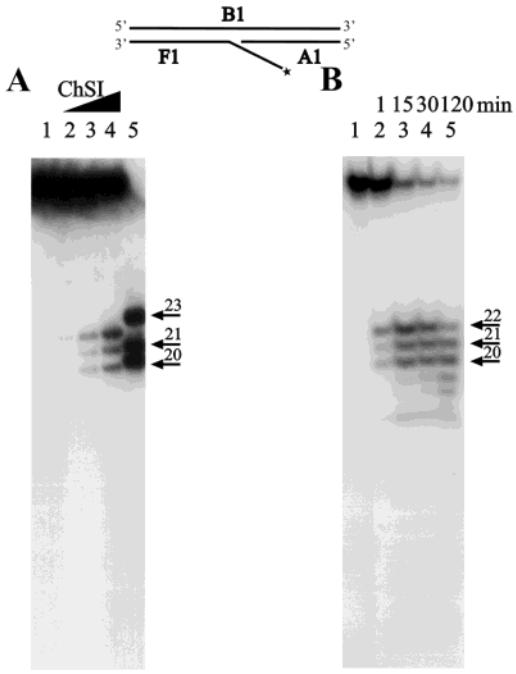


FIGURE 2: ChSI nuclease specifically cleaves the 5′ flap substrate in a structure-specific manner. The reactions were carried out in buffer A. (A) Lane 1: control without enzyme. The 5′ flap substrate was incubated for 1 min with increasing amounts of ChSI (0.04, 0.1, or 0.2 unit, respectively; lanes 2−4). Lane 5: oligonucleotide standards. (B) Lane 1: control without enzyme. The 5′ flap substrate was incubated with ChSI (0.2 unit) for 1, 15, 30, and 120 min (lanes 2−5, respectively). The asterisk indicates the position of the ^{32}P label in F1. The arrows indicate the position and size of the cleavage products and oligonucleotide standards.

to the junction in the double-stranded region, (ii) the 21 nt oligomer, a result of a cut 1 nt proximal to the junction in the double-stranded region, and (iii) the 20 nt oligomer, released by means of the precise cutting of the single-stranded region of 5′ flap DNA. This result indicates that nuclease ChSI recognizes and specifically cleaves 5′ flap branched DNA.

Analyzing the products of a time course reaction (Figure 2B), we found that ChSI cleaves the 5′ flap structure endonucleolytically at the junction between the flap and the double-strand counterpart. The entire 5′ flap oligonucleotide was released as a very early product of the reaction (after 1 min cleavage; see Figure 2B, lane 2). For longer reaction times slow degradation of the flap oligonucleotide downstream of the junction site was observed as a secondary reaction on the primary single-stranded products (Figure 2B, lanes 3−5). Single-stranded oligonucleotides used as controls were fully degraded in an unspecific endonucleolytic manner (data not shown).

To check if a metal cation is necessary for the structure-specific 5′ flap endonuclease activity of nuclease ChSI, the cleavage reaction was performed in the presence of EDTA. The addition of 5 mM EDTA to the reaction mixture almost completely inhibited the enzyme activity (Figure 3A, lane 3), and preincubation of the enzyme with 0.5 mM EDTA had a similar effect (Figure 3B, lane 3). We asked the question, which cation was required for ChSI activity? The enzyme was preincubated with 0.5 mM EDTA for 15 min after which the ability of Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+} cations to restore enzyme activity was examined. Mg^{2+} ion at low concentrations was the most efficient in restoring ChSI activity (Figure 3B, lanes 5 and 6) while the other ions tested were less efficient (Mn^{2+} > Ca^{2+} > Zn^{2+}) (Figure 3C). Interestingly, all the ions tested used at high concentrations (>10 mM) inhibited enzyme activity (Figure 3B, lanes 7 and 8, and Figure 3C). Moreover, addition of divalent ions to the reaction mixture without previous preincubation of the enzyme preparation with EDTA decreased ChSI activity (Figure 4A). The most significant effect of ChSI deactivation was seen for the Mn^{2+} and Zn^{2+} ions used (lanes 6−8 and 12−14, respectively).

ChSI Nuclease Does Not Require Complete 5′ Flap DNA for Efficient Cleavage. It was tempting to ask whether ChSI nuclease requires branched DNA with a complete 5′ flap structure to exhibit efficient structure-specific cleavage. A so-called 5′ pseudoflap DNA was prepared in which the A1 oligonucleotide was deleted from the construct. The cleavage reaction carried out for 2 min with 0.2 unit of ChSI endonuclease resulted in the above-mentioned specific products: the 22, 21, and 20 nt long oligonucleotides (Figure 4B, lane 2). This result showed that the absence of the A1 oligonucleotide did not change the enzymatic cleavage pattern when compared to the complete 5′ flap DNA. Inhibition of the enzyme activity by Zn^{2+}, Ca^{2+}, Mn^{2+}, and Mg^{2+} cations used at high concentrations (up to 10 mM) was also observed. The effect of significant enzyme deactivation by Mn^{2+} and Zn^{2+} ions was similar to that one observed for the 5′ flap branched DNA.

Is the 5′ End of Flap DNA Needed for Specific Recognition by ChSI Nuclease? To answer the question of whether the ChSI nuclease requires a free 5′ end in the 5′ flap DNA, we annealed to the 5′ flap DNA construct a 15 nt primer D1

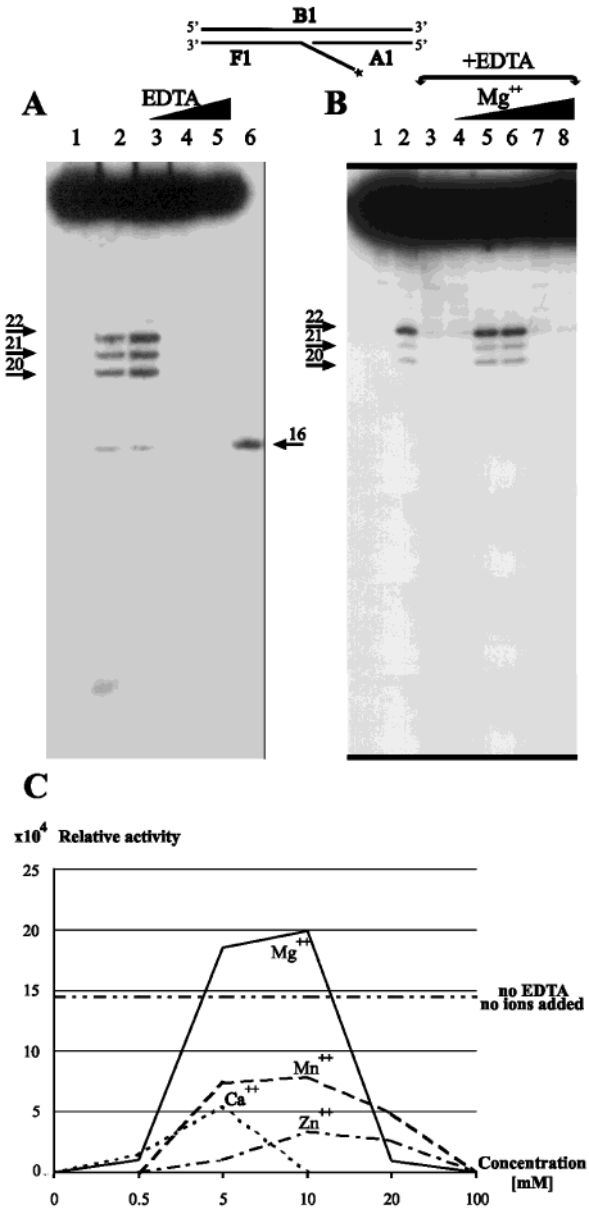


FIGURE 3: ChSI nuclease activity is inhibited by EDTA and restored by Mg^{2+}. (A) The reactions were carried out in buffer A for 1 min. Lane 1: control without enzyme. The 5′ flap substrate was incubated with ChSI (0.2 unit) in the absence (lane 2) or presence of 0.5, 5, or 10 mM EDTA (lanes 3−5, respectively). Lane 6: oligonucleotide standards. (B) ChSI was preincubated with 0.5 mM EDTA in buffer A for 15 min. Mg^{2+} was added and incubation continued for 15 min. The 5′ flap DNA structure was then subjected to the action of ChSI thus prepared for 1 min. Lane 1: control without enzyme. The 5′ flap substrate was incubated with ChSI (0.2 unit) in the absence (lane 2) or presence of 0.5 mM EDTA (lanes 3−8). Lanes 4−8 contained 0.5, 5, 10, 20, and 100 mM Mg^{2+}, respectively. (C) Graphic representation of the quantitative analysis of ChSI activity versus divalent metal ions. Quantitative analysis was performed with ImageQuant software. The relative activity was calculated as the volume created by the 3-D plot of the pixel intensities and the pixel locations within the rectangle.

complementary to the 5′ end of the F1 strand in the 5′ flap substrate. The reactions were performed using increasing concentrations of D1 primer (F1:D1 = 1:0.1, 1:0.5, and 1:1). Previously detected reaction products of 20, 21, and 22 nt were present after addition of D1 primer; however, some inhibition of the enzyme activity was observed, especially

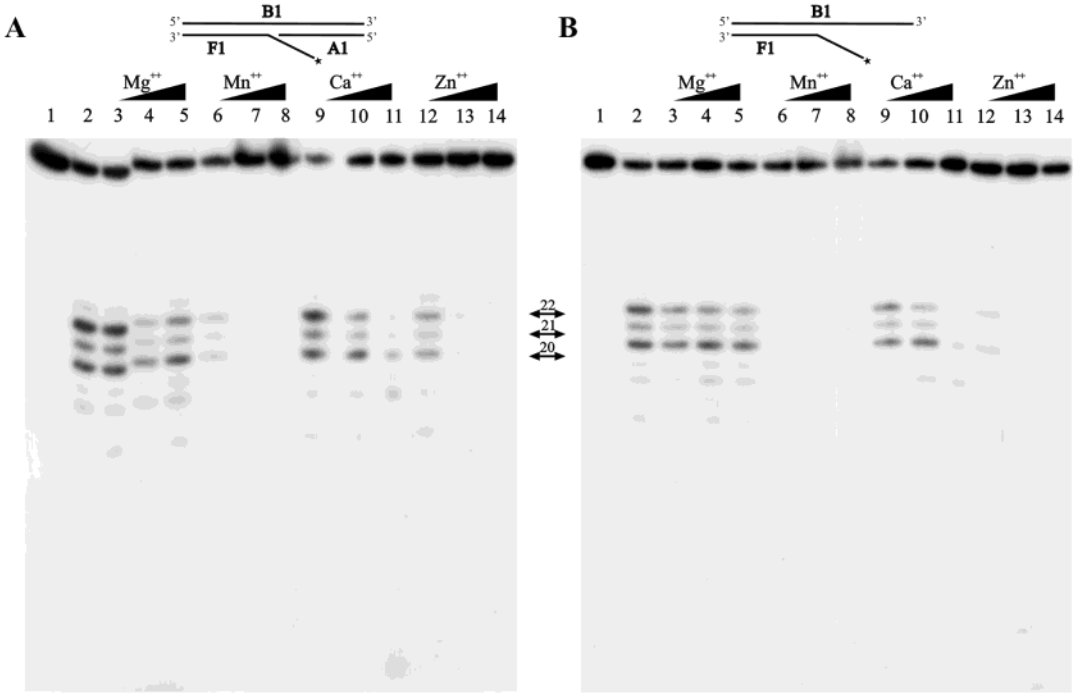


FIGURE 4: Metal ion dependence of ChSI nuclease activity on 5′ flap (A) and 5′ pseudoflap (B) substrates. The reactions were carried out in buffer A for 2 min. (A) Lane 1: control without enzyme. The 5′ flap substrate was incubated with ChSI (0.2 unit) in the absence (lane 2) or presence of 0.5, 5, and 10 mM Mg^{2+} (lanes 3−5), Mn^{2+} (lanes 6−8), Ca^{2+} (lanes 9−11), or Zn^{2+} (lanes 12−14). (B) Lane 1: control without enzyme. The 5′ pseudoflap substrate was incubated with ChSI (0.2 unit) in the absence (lane 2) or presence of 0.5, 5, and 10 mM Mg^{2+} (lanes 3−5), Mn^{2+} (lanes 6−8), Ca^{2+} (lanes 9−11), or Zn^{2+} (lanes 12−14).

with the 1:1 ratio of oligomers (Figure 5, lanes 4 and 5). This result suggests that either a tracking or looping mechanism of the structure-specific cleavage reaction is possible.

ChSI Cleaves Bulged-Out, 3′-Pseudoflap, and 5′ Overhang Single-Stranded DNAs in an Unspecific Manner. We also examined whether nuclease ChSI could act in the absence of a free 5′-terminal oligonucleotide using double-stranded DNA with a 6 nt bulge single strand as a substrate. As shown in Figure 6A, nuclease ChSI released six reaction products, of length 18−23 nucleotides. This experiment showed that the enzyme acts as an unspecific endonuclease toward the single-stranded regions of the used substrates. Divalent metal ions are required for this reaction. Indeed, addition of EDTA to the reaction mixture inhibited ChSI cleaving activity (Figure 6A, lanes 16−18, and Figure 6B). Divalent cations used at high concentrations (10 mM) inhibited, at least to some extent, nucleolytic degradation of the single-stranded region of oligonucleotide B3 (Figure 6A, lanes 4−15).

Also single-stranded regions in 3′-pseudoflap and 5′ overhang DNA constructs were cleaved by ChSI in an unspecific endonucleolytic manner as judged by random distribution of cleavage sites (Figure 7, lanes 4−7 and 10−13, respectively). For comparison see lane 9 in Figure 7, showing that as a control RnI nuclease products resulted from 3′→5′ exonuclease activity of this enzyme.

Neither 5′→3′ nor 3′→5′ Exonuclease Activity Is Associated with ChSI. A duplex of oligonucleotides A1 and B1, annealed before the cleaving reaction, was treated with ChSI, and the cleavage products were analyzed by PAGE. Lanes 2 and 3 of Figure 7 show the result of this experiment. The lack of the radiolabeled 5′-monomer band indicates that the enzyme does not exhibit 5′→3′ exonucleolytic activity typical

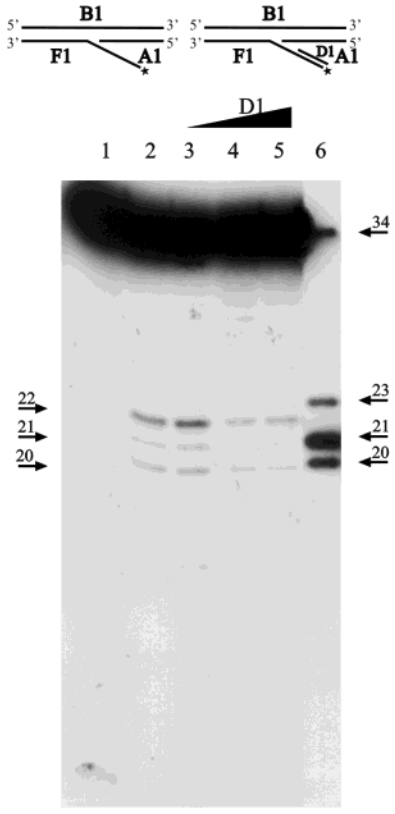


FIGURE 5: Effect of hybridization of a primer to the single-stranded 5′ tail in the 5′ flap substrate on ChSI nuclease activity. The reactions were carried out in buffer A for 2 min. Lane 1: control with neither enzyme nor D1 primer. The 5′ flap substrate was incubated with ChSI (0.2 unit) in the presence of increasing amounts of D1 primer in the following ratios to F1 (0:1, 0.1:1, 0.5:1, and 1:1, respectively; lanes 2−5). Lane 6: oligonucleotide standards.

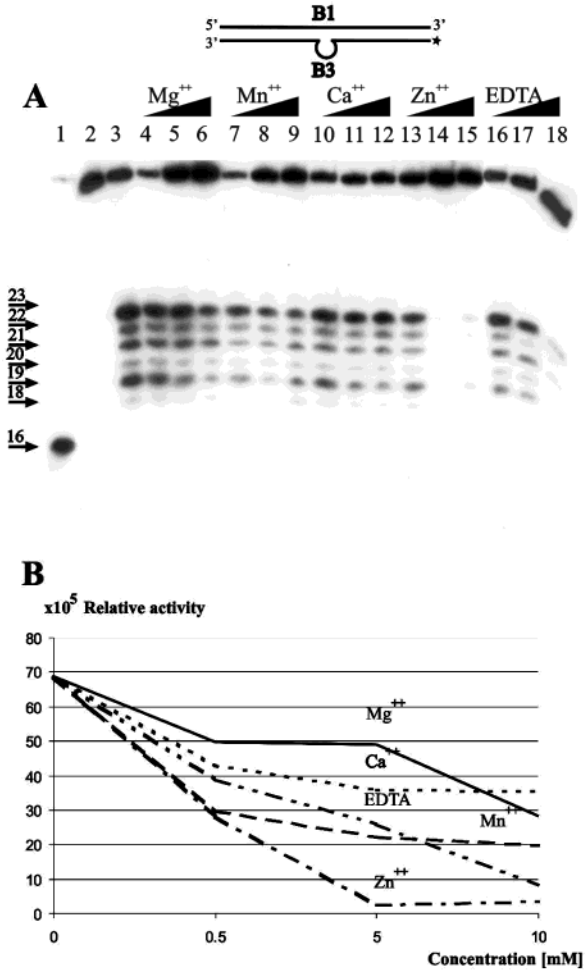


FIGURE 6: Metal ion dependence of ChSI nuclease on a bulged-out structure. The reactions were carried out in buffer A. (A) DNA containing a 6 nt bulge was incubated for 1 min with ChSI (0.2 unit) in the absence (lane 3) or presence of 0.5, 5, and 10 mM Mg^{2+} (lanes 4−6), Mn^{2+} (lanes 7−9), Ca^{2+} (lanes 10−12), Zn^{2+} (lanes 13−15), or EDTA (lanes 16−18). Lanes: 1, oligonucleotide standards; 2, control without enzyme. (B) Graphic representation of the quantitative analysis of ChSI activity versus divalent metal ions (the intensity of the 23 nt band was counted). Quantitative analysis was performed with ImageQuant software as in Figure 3.

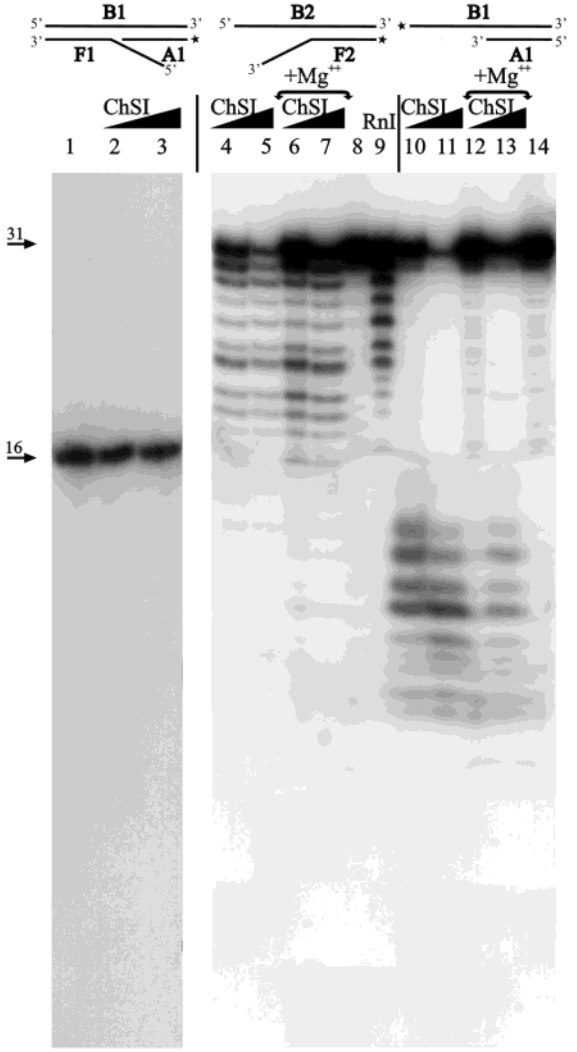


FIGURE 7: Analysis of ChSI substrate specificity. The reactions were carried out in buffer A. Lanes: 1−3, double-stranded blunt ends; 4−9, 3′ pseudoflap substrate; 10−14, 5′ overhang substrate. DNA substrates were incubated with 0.1−0.2 unit of ChSI in the absence of Mg^{2+} (lanes 2−3, 4−5, 10−11) or presence of 10 mM Mg^{2+} (lanes 6−7, 12−13). Lanes 1, 8, and 14: controls without enzyme. Lane 9: control with RnI nuclease (0.2 unit).

for all other 5′ flap nucleases. To be sure that the absence of the monomer band is not due to the fact that small products have simply migrated off the gel, additional experiments were carried out using MALDI-TOF mass spectrometry (see following paragraph).

Nucleolytic Properties of ChSI toward Single- and Double-Stranded DNA As Analyzed by MALDI-TOF Mass Spectrometry. MALDI-TOF mass spectrometry was used to monitor the nucleolytic activity of nuclease ChSI toward single- or double-stranded DNA. Complementary octamers 5′-d(GCAAAAGC)-3′ (C1, MW 2427) and 5′-d(GCTTTTGC)-3′ (C2, MW 2391) and their 1:1 molar ratio complex (C1/C2) were digested with nuclease ChSI under typical reaction conditions, except that the experiments were carried out at 0 °C instead of 25 or 37 °C, due to the thermodynamic stability of the C1/C2 duplex. The T_m of this complex is 34 °C as determined by UV melting measurements. Aliquots were withdrawn after 10, 30, 60, 120, and 240 min, and the resulting cleavage products were analyzed by MALDI-TOF mass spectrometry. The reaction times were much longer that those used for the cleavage of the 5′ flap DNA construct, and the concentration of the ChSI enzyme was much higher to ensure proper interpretation of the MALDI-TOF results.

Figure 8A,B shows the mass spectra (negative ions) of cleavage products of oligonucleotides C1 and C2, respectively, obtained after 120 min. Both oligonucleotides tested are predominantly cleaved in the central domains of the parent sequences. The major products of endonucleolytic cleavage of oligonucleotide 5′-d(GCAAAAGC)-3′ are oligomers 5′-d(GCA)-3′ (*m*/*z* 869), 5′-d(GCAA)-3′ (*m*/*z* 1181), and 5′-d(GCAAA)-3′ (*m*/*z* 1495) and the remaining 5′-phosphorylated 3′-terminal oligomers 5′-d(pAAAGC)-3′ (*m*/*z* 1575), 5′-d(pAAGC)-3′ (*m*/*z* 1262), and 5′-d(pAGC)-3′ (*m*/*z* 949), respectively (Figure 8A). The analogous signal pattern of the digestion products of oligonucleotide C2 is seen in Figure 8B. The diagrams of the corresponding *m*/*z* values of C1 and C2 cleavage products are shown in panels A and B of Figure 9, respectively. Lower molecular weight products that arise from further nucleolytic degradation of C1 and C2

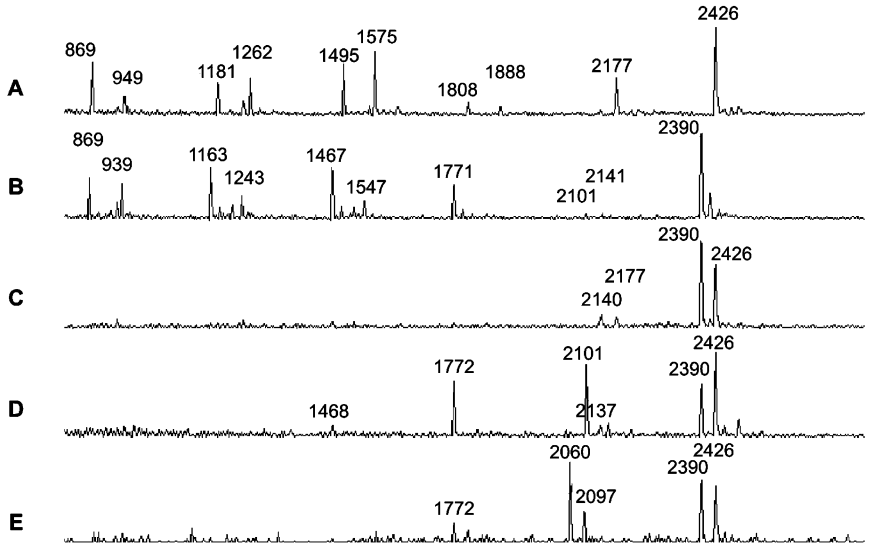


FIGURE 8: Cleavage activity of nuclease ChSI toward single- and double-stranded DNA. The MALDI-TOF spectra of the degradation products of oligonucleotides C1 [5′-d(GCAAAAGC)-3′] (A) and C2 [5′-d(GCTTTTGC)-3′] (B) with ChSI and the 1:1 complex of C1 and C2 with nuclease ChSI (C), the C1/C2 complex with snake venom PDE I (D), and calf spleen PDE II (E) are shown. Cleavage reaction conditions are given in Materials and Methods.

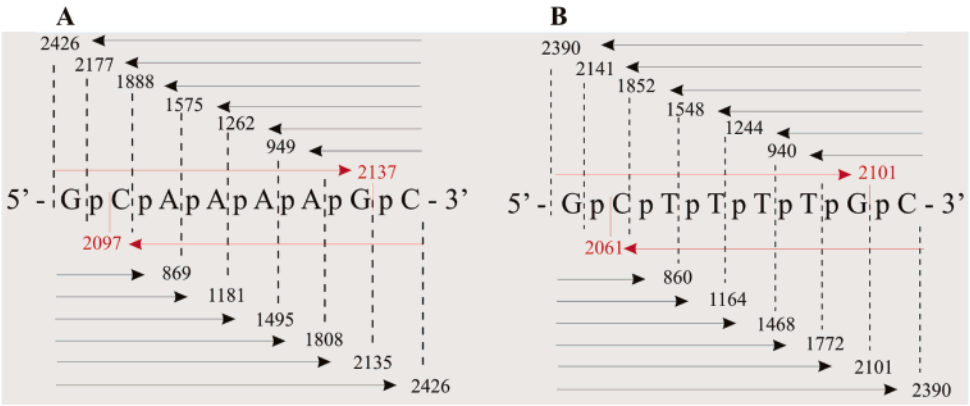


FIGURE 9: Diagram of the nucleolytic degradation products of oligonucleotides C1 (A) and C2 (B). The products of the initial 3′- and 5′-exonucleolytic cleavage of the parent oligodeoxyribonucleotides are shown in red.

are not shown. Notably, in both series of MALDI-TOF mass spectra the intensities of the ions representing the cleavage products of C1 and C2 are virtually identical regardless of the duration of the reaction (see Supporting Information). Such pattern of peaks is attributed to endonucleolytic degradation of the parent oligonucleotides.

Double-stranded DNA formed by annealing equimolar amounts of oligonucleotides C1 and C2, when subjected to the above degradation reaction (120 min), does not result in similar degradation products. The only signals seen in the C1/C2 duplex spectrum (Figure 8C), apart from those corresponding to the parent oligonucleotides C1 (*m*/*z* 2426) and C2 (*m*/*z* 2390), are the signals of minute products formed by endonucleolytic removal from each strand of the last 5′-terminal deoxyribonucleoside. A comparison of the cleavage products of C1 and C2 with those released from digestion of the C1/C2 complex clearly indicates that nuclease ChSI cleaves exclusively single-stranded DNA but not double-stranded DNA. In contrast, nuclease P1, used as control, digested the C1/C2 complex with high efficiency, leading to 5′-phosphate deoxyribonucleotides (see Supporting Information).

To prove that nuclease ChSI does not exhibit 3′- or 5′-exonucleolytic activity, the C1/C2 duplex was subjected to nuclease ChSI (Figure 8C) and in parallel control experiments to exonucleolytic degradation with snake venom phosphodiesterase (PDE I), which is a 3′-exonuclease (Figure 8D), and with calf spleen phosphodiesterase (PDE II) exhibiting 5′-exonuclease activity (Figure 8E). In the MALDI-TOF mass spectra recorded for the two latter experiments there are signals originating from the products that are gradually released by exonucleolytic cleavage of each strand of the duplex. The 3′-exonuclease cleaves oligonucleotide C1 to yield products of *m*/*z* 2101 (5′-GCTTTTG-3′, marked in red in Figure 9B), 1772 (5′-GCTTTT-3′), and 1468 (5′-GCTTT-3′). Oligonucleotide C2 is cleaved with lower efficiency, and a product of *m*/*z* 2137 (5′-GCAAAAG-3′, marked in red in Figure 9A) is released. Longer incubation times result in further exonucleolytic cleavage of both DNA strands yielding products of lower molecular weight (see Supporting Information). Similarly, the 5′-exonuclease PDE II cleaves both oligonucleotides of the C1/C2 duplex from their 5′ ends. The 5′-terminal nucleotide is removed from each strand, giving products of *m*/*z* 2097 (5′-CAAAAGC-3′) and of *m*/*z* 2060 (5′-CTTTTGC-3′), respectively. Further

Table 3: Kinetic Parameters of Nucleolytic Activities of ChSI

	5′ flap-specific endonuclease activity				single-strand-specific endonuclease activity			
product	K_M (nM)	V_{max} (nM/min)	k_{cat} ($\times 10^3$ min^{-1})	k_{cat}/K_M ($\times 10^4$ min^{-1} nM^{-1})	K_M (nM)	V_{max} (nM/min)	k_{cat} ($\times 10^3$ min^{-1})	k_{cat}/K_M ($\times 10^4$ min^{-1} nM^{-1})
22 nt	4.1	0.170	1.30	28.00	13.2	0.78	0.70	0.50
21 nt	2.1	0.054	0.43	7.00	20.1	0.81	0.99	0.47
20 nt	2.1	0.046	0.34	2.00	34.4	1.64	1.50	0.46

similar degradation of both oligonucleotides is observed on prolonged reaction times (see Supporting Information). It is noteworthy that with ChSI in the MALDI-TOF spectra of C1, C2, or the corresponding duplex there are no signals of *m*/*z* 2060 and 2097, representing products after removal from the parent strand of the 5′-terminal Gp nucleotide, that could be attributed to 5′-exonucleolytic activity of nuclease ChSI. Also, 3′-exonucleolytic activity of the ChSI enzyme can be excluded on the basis of the lack of the characteristic patterns of peaks in Figure 8A−C, as in Figure 8D,E, representing high-intensity peaks of the substrates shortened by one nucleotide (5′-GCAAAAG-3′and 5′-GCTTTTG-3′), while intense peaks of products released by the cleavage within the central domains of the parent single-stranded oligomers are present (as in Figure 8A,B). Also, as mentioned above, very similar intensity of ions corresponding to ChSI cleavage products of single-stranded DNA, independent of the reaction time (see Supporting Information), indicates that nuclease ChSI does not possess 3′-exonucleolytic activity.

ChSI Cleavage Kinetics. We have determined the cleavage parameters of the ChSI nuclease with respect to the 5′ flap substrate and to the single-stranded oligonucleotide F1. Michaelis−Menten kinetic analyses were used to derive the V_{max}, K_M, and k_{cat} values. The amount of ChSI enzyme used to cleave the F1 oligomer was 8-fold greater than that used for the cleavage of the 5′ flap substrate. The intensities of the three specific products, oligomers 22, 21, and 20 nt, were evaluated for reactions performed on both substrates. Kinetic data (Table 3) reveal that the affinity of nuclease ChSI for the 5′ flap substrate is remarkably higher than for oligonucleotide F1; the K_M values for the 5′ flap substrate are lower by factors of 3.2 (22 nt), 9.6 (21 nt), and 16.3 (20 nt), compared with the respective values for the F1 substrate. The k_{cat} value is higher for the 5′ flap substrate only for the cleavage reaction releasing the 22 nt product. However, the catalytic efficiency (k_{cat}/K_M) of the enzyme is significantly higher for the 5′ flap than for the single-stranded substrate. The k_{cat}/K_M values decrease 56-fold (22 nt), 15-fold (21 nt), and 4.3-fold (20 nt) for the nonspecific endonucleolytic activity.

We asked the question whether the presence of magnesium ions affects substrate binding affinity or changes the rate of catalytic activity of the ChSI enzyme. Kinetic parameters for the 5′ flap substrate were determined in the reaction with no magnesium ions added or with 2.5 and 10 mM Mg^{2+} (Table 4). The magnesium-dependent catalytic activity of the 5′ flap-specific endonuclease ChSI has the following characteristics. For each reaction, regardless of the length of the product, the K_M values are lowest in the presence of 2.5 mM magnesium ion. Increased Mg^{2+} concentration causes significant lowering of the enzyme affinity for its substrate. V_{max} values do not differ significantly regardless of the length of the released product and the divalent ion concentration. Interestingly, the catalytic efficiency of the enzyme in each reaction is highest for 2.5 mM magnesium ion. These results are consistent with previous findings that the activities of known plant 5′ flap nucleases are stimulated by Mg^{2+} ions at physiological concentrations (*47*). However, at higher concentrations, divalent cations inhibit specific cleavage.

Table 4: Magnesium-Dependent Catalytic Activity of 5′ Flap-Specific Endonuclease ChSI

product	added Mg concn (mM)	K_M (nM)	V_{max} (nM/min)	k_{cat} ($\times 10^3$ min^{-1})	k_{cat}/K_M ($\times 10^4$ min^{-1} nM^{-1})
22 nt	0.0	1.007	0.0174	0.126	1.25
	2.5	0.67	0.0058	0.096	1.43
	10.0	4.7	0.0155	0.108	0.23
21 nt	0.0	0.78	0.0060	0.042	0.54
	2.5	0.17	0.0055	0.036	2.12
	10.0	8.14	0.0179	0.126	0.15
20 nt	0.0	0.88	0.0059	0.042	0.48
	2.5	0.12	0.0052	0.036	3.00
	10.0	4.76	0.0109	0.078	0.16

The summary of the ChSI nuclease activities on different synthetic DNA substrates is presented in Table 2.

DISCUSSION

Many important pathways of DNA metabolism involve the transient formation of branched DNAs that contain free 5′ single-stranded ends. DNAs with such structures can arise during replication (*1*), recombination (*6*, *7*), and repair processes (*4*). These bifurcated molecules are trimmed by a group of homologous 5′ flap nucleases also known as 5′→3′ exonucleases (*8*). Chloroplasts, the site of photosynthesis, are plant organelles that possess their own DNA; they are exposed to sunlight, including UV which is known to cause DNA damage (*41*). The mutagenic effect of the numerous redox reactions and electron transfer processes that occur in chloroplasts also affects DNA (*32*). It seemed interesting to check if the ChSI nuclease isolated from wheat chloroplasts (*28*) might possess the activity of 5′ flap nuclease and therefore participate in the repair process of chloroplast DNA. Indeed, here we demonstrate that ChSI has a strong 5′ flap structure-specific nuclease activity. The results obtained suggest that ChSI may be the plant counterpart of the 5′ flap family of nucleases that are conserved from phages to humans (*18*).

The structure-specific cleavage reaction of known 5′ flap nucleases such as FEN-1 usually require divalent metal cations (*9*). The activities of the known plant 5′ flap nucleases (from cauliflower influorescence and from rice) are stimulated by Mg^{2+} and Mn^{2+} (*26*, *27*). ChSI, which also requires Mg^{2+} or Mn^{2+}, binds its cofactor fairly tightly (in the micromolar range), and therefore there is no need to add

cations to the digestion reactions carried in vitro. Moreover, higher concentrations of divalent cations (in the millimolar range) inhibit specific cleavage.

Two possible mechanisms of the 5′ flap enzymatic reaction have been proposed to date: either a tracking (sliding; *42*−*45*) or looping mechanism (*46*). For example, it has been shown that the calf 5′→3′ exo/endonuclease (the counterpart of the human FEN-1 and yeast RTH-1 nucleases) must enter by way of the tail at the 5′ end of the flap and slide along the DNA up to the point of cleavage. An alternative to tracking on the single-stranded tail is that the enzyme needs only to bind to or associate with the 5′ end of the tail to be activated. In the activated state, it could then bind directly to the point of cleavage and start hydrolysis (looping mechanism; *46*). Since we observed some inhibition of the reaction by annealing of primer D1 complementary to the 5′ end of the flap (Figure 5), either the tracking or looping mechanism is possible for 5′ flap structure-specific ChSI cleavage.

ChSI nuclease also acts as an unspecific endonuclease cleaving single-stranded regions such as bulges, 5′ overhangs, or 3′ pseudoflaps. In this case the nuclease does not recognize a specific junction point between the single- and double-stranded DNA but hydrolyzes any phosphodiester bond in the single-stranded region, cleavage occurring with the highest probability in the middle of this region. Presumably ChSI cleaves 5′ flap DNA and other single-stranded fragments using the same active site, because in both cases we observed the same dependence of activity on the presence of various divalent cations.

We have also shown that ChSI nuclease does not require a complete 5′ flap structure and efficiently cleaves 5′ pseudoflap structures deprived of A1 primer.

The characteristics of the 5′ flap structure-specific ChSI nuclease activity show that the chloroplast enzyme is different from other known 5′ nucleases including plant enzymes from cauliflower influorescence (26) or from rice (27), because, as opposed to other known 5′ nucleases, ChSI does not show any 5′→3′ exonuclease activity on double-stranded substrates. It is worth stressing that for ChSI in the MALDI-TOF spectra of C1, C2, or their complementary duplex there are no signals of *m*/*z* 2060 or 2097, representing products after removal of the 5′-Gp units from the parent oligonucleotides, that could be attributed to 5′-exonucleolytic activity of nuclease ChSI. Also, 3′-exonucleolytic activity of the ChSI enzyme can be excluded on the basis of the very similar intensity pattern of ions corresponding to shortened oligonucleotides, independently of the time of the reaction as mentioned above. Also, in contrast to other known plant 5′ flap nucleases (*26*, *27*), ChSI has high unspecific endonucleolytic activity toward single-stranded DNA as evidenced by both MALDI-TOF mass spectrometry and polyacrylamide gel electrophoresis. The specificity of ChSI nuclease for these substrates is, however, much lower than for the 5′ flap substrates. The binding affinity of the 5′ flap DNA is at least several times higher than for single-stranded DNA. Moreover, the catalytic efficiency (k_{cat}/K_M) of the enzyme is significantly higher for the 5′ flap than for the oligonucleotide F1. These data suggest that ChSI nuclease possesses both activities, with a preference for 5′ flap-specific endonucleolytic cleavage. This activity is preferred at a rather low magnesium concentration, and this is consistent with the findings that the activities of the known plant 5′ flap nucleases are stimulated by Mg^{2+} ions at physiological concentration (*47*). However, at higher concentrations, the divalent cations Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+} inhibit specific cleavage. Although the values of the catalytic efficiency of ChSI are much lower as compared to human FEN-1 (*21*) and HEX1-N2 (*48*) as well as for *M. jannaschii* FEN-1 data (*21*), they are similar to the values determined for murine (*47*), *P. furiosus* (*21*) and *A. fulgidus* FEN-1 enzymes (*21*). The k_{cat}, K_M, and catalytic efficiency values of several 5′ flap endonucleases are compared in Table 5. It is possible that our enzyme is similar to other 5′ flap nucleases identified in fungal mitochondria (*Podospora anserina*), which also exhibit nonspecific endonucleolytic activity toward single-stranded DNA substrates (*48*). It can be that the nonspecific endonucleolytic acitvity of 5′ flap endonucleases toward single-stranded DNA substrates is a cognate feature of enzymes present in plants and fungi organelles. It is difficult to deduce unequivocally the true function of the magnesium ions in the ChSI nucleolytic activity. Our results show that magnesium ions are essential for enzyme activity (5 mM EDTA completely removes enzyme activity, and addition of Mg^{2+} restores it). However, the dissociation constant is somehow dependent on the Mg^{2+} ions and is lowest (the best binding between the 5′ flap substrate and the endonuclease) at 2.5 mM Mg^{2+}. In high Mg^{2+} concentrations the enzyme loses its activity, and this may suggest reorganization of the protein to a nonactive conformation.

Table 5: Comparison of Kinetic Parameters for Selected 5′ Flap Endonucleases

source	enzyme	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} nM^{-1})	ref
T. vulgare	ChSI	4.1[a]	0.0013	0.0028	
Homo sapiens	hFEN-1	0.40[b]	0.91	2.3	*21*
A. fulgidus	aFEN-1	0.41[b]	0.0052	0.013	*21*
M. jannaschii	mFEN-1	0.38[b]	0.018	0.48	*21*
P. furiosus	pFEN-1	0.17[b]	0.00068	0.004	*21*
murine	MFEN-1	89.3[c]	0.09	0.001	*47*
H. sapiens	HEX1-N2	8.0[d]	2.73	0.34	*48*

[a] 20 mM Tris-HCl, pH 7.5. [b] 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 50 μg/mL bovine serum albumin. [c] 50 mM Tris-HCl, pH 8.0. [d] 20 mM HEPES, pH 7.5, 50 mM KCl, 0.5 mM DTT, 5 mM $MgCl_2$, 0.05% Triton X-100, and 100 μg/mL 5% glycerol.

Therefore, we conclude that the 5′ flap endonuclease ChSI from chloroplasts belongs to a subclass of 5′ nucleases that has so far not been described.

SUPPORTING INFORMATION AVAILABLE

MALDI-TOF spectra of the degradation products of C1 and C2 and their equimolar complex with each of the nucleases tested (ChSI, svPDE, calf spleen PDE and P1) after at least four different digestion reaction times plus reference spectra of C1 and C2. This material is available free of charge via the Internet at http://pubs.acs.org.

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