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Direct evidence for the deoxyribonuclease activity of the plant ribosome inactivating protein gelonin

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Abstract Several plant ribotoxins, including gelonin, were reported to have additional weak nuclease activities on supercoiled DNA. The potential contribution of this activity to their cytotoxicity has not been given serious consideration due to concerns about contaminating nucleases in the protein preparations. We now report the degradation of single-stranded DNA by preparations of native plant gelonin and recombinant gelonin produced in *E. coli*. The DNase activity of both preparations is similarly modulated by zinc. An SDS-PAGE DNase assay identifies gelonin as the polypeptide responsible for deoxyribonuclease activity.

Key words: Gelonin; Nuclease; Single-stranded DNA; Zinc

1. Introduction

Ricin and other related plant proteins such as abrin, gelonin, trichosanthin or pokeweed antiviral protein (PAP) have been classified as RIPs (ribosome inactivating proteins) or ribotoxins to refer to the fact that they are cytotoxic by inactivation of the ribosomes [1]. The molecular mechanism of inactivation has been elucidated in a cell-free system by Endo and colleagues [2]: plant RIPs remove an invariant adenine in a conserved loop of the 28S rRNA, rendering the 60S subunit unable to bind EF-2 effectively, thereby inhibiting protein synthesis.

Additional in vitro activities of ribotoxins against both RNA and DNA have been described in recent years. Stirpe and colleagues determined that some RIPs remove adenine from rRNA at more than one site [3]. More recently, the same authors described the release of multiple adenines from different RNAs as well as DNA by the RIP saporin L-1 [4,5]. Lee-Huang and colleagues were among the first to report that ribotoxins, including gelonin, nicked and linearized supercoiled DNA [6]. Similar action on DNA has now been described for a number of RIPs [7,12]. In all cases, a high protein to DNA ratio, e.g. 4:1 (w/w), was necessary to reveal the DNase activity. In a very recent study [13], it has been suggested that the RIPs dianthin, saporin 6 and gelonin are single-stranded DNases that cleave supercoiled DNA at the A+T rich regions which are partially single-stranded due to supercoiling. This result is at odds with a previous publication that failed to detect nuclease activity of RIPs on single-stranded M13 DNA [12].

The identification and characterization of the DNase activity of ribotoxins may provide a clue in determining their role in plant physiology. Moreover, the action on DNA could be responsible for the anti-viral activities of ribotoxins which do not appear to be solely due to inhibition of protein synthesis [4,5,14–16]. It is essential to establish that additional activities are inherent properties of the ribotoxins and not due to the presence of contaminants in the protein preparations. In the present study, we report that native and bacterial-recombinant gelonin exert nuclease activity on single-stranded DNA.

2. Material and methods

2.1. Materials

Plant gelonin (nGel) was a gift from Dr. Fiorenzo Stirpe [17]. Recombinant gelonin (rGel) was a gift from Dr. Steve Carroll [18]. A polyclonal anti-gelonin antibody was a gift from Dr. Vinod Singh [19]. The pUC18 DNA plasmid and the 100 bp ladder were obtained from Gibco BRL (Grand Island, NY). Calf thymus single-stranded DNA was from the Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was from Boehringer-Mannheim Corp. (Indianapolis, IN). Trevigel™ 500 Orange G loading buffer were from Trevigen (Gaithersburg, MD). The silver stain kit was Gelcode from Pierce (Rockford, IL). The nitrocellulose Hybond c and the ECL Western blotting detection kit from Amersham Life Sciences, Inc. (Arlington Heights, IL). The low molecular mass electrophoresis calibration kit from Pharmacia Biotech (Piscataway, NJ) was used for molecular mass markers. Tris buffers were prepared by dilution of a DNase, RNase-free 1 M stock solution (Sigma) with autoclaved water.

2.2. DNase assay

Linear pUC18 DNA was prepared by incubation with *Hin*dIII, phenol/chloroform extraction and ethanol precipitation. The concentration was determined by absorption spectroscopy. Single-stranded DNA was prepared by boiling for 2 min and rapid cooling. DNA (0.5 μg) was incubated with or without 1 μg of nGel or rGel in 10 mM Tris, pH 8.0, in the presence of various concentrations of zinc sulfate (0–2 mM) in a total volume of 20 μl. After 1 h at 37°C, 4 μl of Orange G loading buffer was added. Electrophoresis was carried out under non-denaturing conditions in a 1% Trevigel™ 500 gel and the DNA visualized by ethidium bromide staining.

2.3. Zymography

The analysis was performed essentially as described in [20] with modifications in the development step as suggested in [21]. Protein samples were mixed with 0.1 M sodium phosphate, pH 6.0, 1% SDS, 10% glycerol, 2% β-mercaptoethanol (βME), 0.01% bromphenol blue and run in duplicate on an 11% polyacrylamide gel containing 1 μg/ml of single-stranded calf thymus DNA. After electrophoresis, one half of the gel was developed by silver staining to reveal the proteins. SDS was removed from the other half of the resolving gel by immersion in 10 mM Tris-HCl, pH 7.4, 5 mM βME, 25% isopropanol. The gel was rinsed in 10 mM Tris-HCl, pH 7.4, incubated overnight at 4°C in 50 mM Tris-HCl, pH 7.4, for protein renaturation and for an additional 48 h in 10 mM Tris-HCl, pH 8.0, 5 mM βME, 1.5 mM zinc sulfate at 37°C. For a negative control, zinc was omitted. Before development of the zymogram by silver staining, the proteins were denaturated by soaking the gel for 1 h in 10% SDS and electroblotted onto nitrocellulose using a Bio-Rad transblot apparatus. The transfer was performed at 100 V for 1 h. To enhance the DNA staining, the aldehyde and base solutions were diluted 1:22 with

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water. The nitrocellulose sheet was processed for ECL Western blot analysis with the anti-gelonin antibody.

3. Results

When nGel and rGel were tested for their activity on supercoiled or double-stranded (ds), linear DNA, the results (not shown) were found to be in accordance with previous reports that described a low (magnesium-independent) activity of various RIPs to nick and linearize supercoiled DNA and no activity against linear, dsDNA [7–13]. Fig. 1 shows the effect of nGel (A) and rGel (B) on single-stranded (heat-denatured) DNA at a ribotoxin/DNA ratio of 2:1. In the absence of any added divalent cations (lane 1), these proteins degraded the DNA into fragment of different size as evidenced by the appearance of a smear. DNA damage was detected at nGel or rGel/DNA ratios as low as 1:1 (data not shown). When the incubation was performed in the absence of toxin (lane C), the DNA was not degraded, indicating that neither the buffer nor the DNA preparation contained contaminating nucleases.

Since gelonin has been reported to bind zinc [22] and zinc modulates the activity of single-stranded (ss) DNases [23], the effect of this cation was investigated (Fig. 1, lanes 2–4). As evidenced by the shift in the position of the smear, a strong zinc dependence of the ssDNase activity of nGel and rGel was observed. The addition of 0.01 mM Zn^{+2} similarly stimulated the activity of nGel and rGel, causing total degradation of the DNA into smaller fragments. Maximum activity (e.g. the production of smaller, faster migrating DNA fragments) was observed when the incubation included 0.1 mM Zn^{+2}. Zinc concentrations ≥1.0 mM were inhibitory; 2.0 mM zinc completely inhibited the DNase activity. Zinc alone (0.1–2.0 mM) did not cause the degradation of the DNA (data not shown). Thus, zinc modulated the degradation of ssDNA by nGel and rGel, with a maximum activity obtained in the presence of 0.1 mM zinc for both preparations.

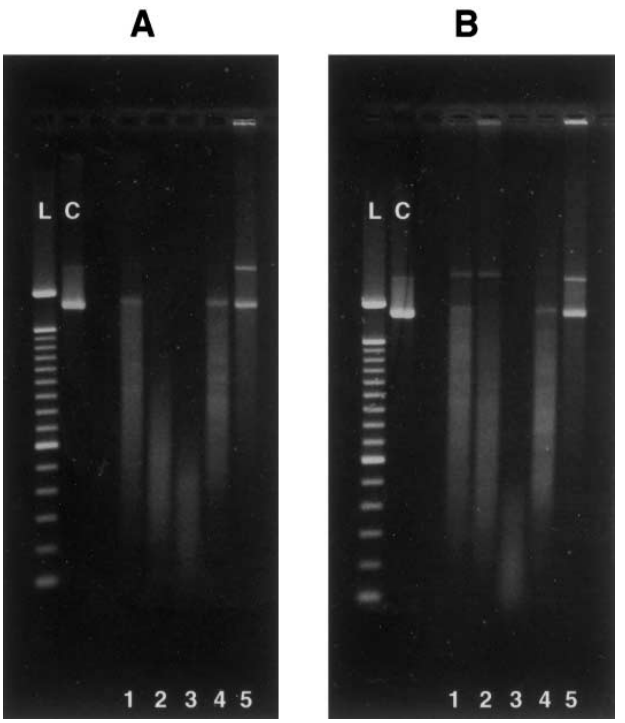

Fig. 1. The modulation of nGel and rGel single-stranded nuclease activity by zinc. Following preincubation for 30 min in 10 mM Tris-HCl, pH 8.0, with various concentrations of $ZnSO_4$ (0, 0.01, 0.1, 1.0 and 2.0 mM, lanes 1–5), plant (A) and recombinant (B) gelonin were incubated with single-stranded pUC18 DNA (0.5 μg) for 1 h at 37°C. The samples were analyzed by electrophoresis in 1% Trevigel. L, 100 bp ladder; C, 0.5 μg of DNA incubated as above in the absence of gelonin.

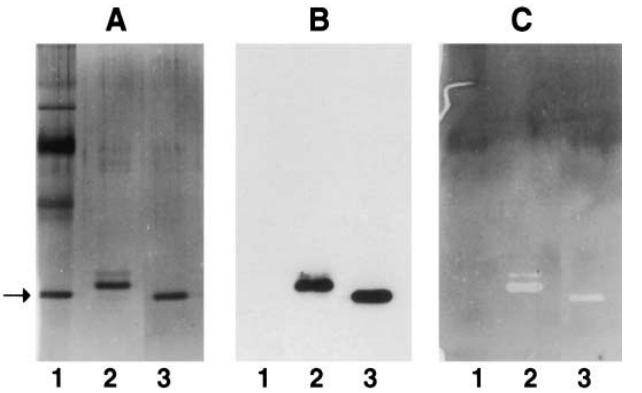

Fig. 2. Zymogram identifying gelonin as the polypeptide responsible for the DNase activity. Characterization of nGel and rGel by (A) protein silver staining, (B) Western blotting with an anti-gelonin antibody and (C) DNA silver staining after transfer of the proteins to nitrocellulose. Lane 1 of each panel contains 0.3 μg of molecular mass markers (M) and lanes 2 and 3 contain 0.1 μg of nGel and rGel, respectively. The arrow indicates the position of the 30 kDa molecular mass marker.

In order to identify the polypeptide responsible for the ssDNase activity, a zymographic analysis of nGel and rGel was performed. The proteins, which were separated in a SDS-PAGE gel containing single-stranded DNA, were incubated in the presence of zinc after renaturation. Development of the zymogram with ethidium bromide, revealed dark bands against a fluorescent background at the expected molecular mass of nGel and rGel, which could have been interpreted as due to the nuclease activity of these proteins. However, their disappearance when the proteins were denatured by soaking the gel in SDS (data not shown), suggested they were 'false-positive' signals, probably due to the exclusion of ethidium bromide, similar to a phenomenon described for histones [21,24]. In order to avoid this artifact, the presence of the DNA in the gel was detected by silver staining after electrotransfer of the proteins onto a nitrocellulose membrane [21]. With this assay, true nuclease activity appears as a clear band on a brown background of stained DNA [21]. Fig. 2C shows a zymogram developed under these conditions. The nuclease activity of the nGel preparation (lane 2) was clearly evidenced by the two clear bands. They migrated at the same positions as the bands detected by protein staining of nGel before transfer (Fig. 2A) which were identified as gelonin isoforms [25] by Western blotting (Fig. 2B). The nuclease activity of the preparation of rGel (lane 3) also migrated to the same position as the band identified as gelonin with the antibody. The molecular mass markers (lane 1) provided a negative control, revealing that DNA-free regions (bands) were not an artifact caused by the exclusion of DNA from areas in the gel occupied by any protein. No signal was detected when the zinc was omitted from the incubation buffer (data not shown).

4. Discussion

A number of investigations have suggested that plant ribotoxins possess a DNase activity in addition to their well-established ability to remove an invariant adenine from rRNA. The validity of these findings has been questioned due to concerns about nuclease contamination of the preparations. A classical approach to address the issue of contamination is to compare the activities present in preparations of natural and recombinant proteins because it is unlikely that they could contain the same contaminant. In this study, we identified a novel activity of native plant gelonin, namely, the zinc-activated degradation of single-stranded DNA, and found that recombinant gelonin exhibited the same characteristics. Moreover, we identified the polypeptides responsible for the DNase activity of the two preparations by in situ detection after SDS-PAGE. As three bands of different molecular mass, all identified as gelonin by Western blotting, were detected by the SDS-PAGE DNA assay, it can be concluded that the nuclease activity is associated with gelonin because the likelihood that three contaminating nucleases co-migrated with the three forms of gelonin is extremely remote.

Future investigations will determine if the zinc-stimulated degradation of single-stranded DNA is a general property of plant RIPs. If so, the questions of their biological function and the mechanism of their anti-tumor and anti-viral properties will have to be re-evaluated in the light of this additional property. In a recent study, one argument to dismiss the in vivo role of the DNase activity of a RIP has been that the cytotoxic concentration was 100-fold less than the concentration required to detect the cleavage of DNA in vitro [26]. We and others [13] have shown that, with modifications of the reaction conditions, DNA damage can be detected in vitro at a 10- to 50-fold lower gelonin/DNA ratio than previously described. Moreover, it is difficult to predict the effects the DNase activity may produce inside a (virus-infected) cell. A more definitive answer will require the analysis of host and viral DNAs in ribotoxin intoxicated cells.

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