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Isolation of a Novel Deoxyribonuclease with Antifungal Activity from *Asparagus officinalis* Seeds

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A deoxyribonuclease distinct from the previously isolated asparagus ribosome-inactivating proteins, possessing a molecular weight of 30 kDa and requiring a pH of 7.5 for optimum hydrolytic activity toward herring sperm DNA, was isolated from *Asparagus officinalis* seeds. The isolation procedure involved extraction with saline, $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on DEAE–cellulose, affinity chromatography on Affi-gel blue gel, ion-exchange chromatography on CM-Sepharose, and FPLC gel filtration on Superdex 75. The doxyribonuclease was unadsorbed onto DEAE–cellulose and Affi-gel blue gel and adsorbed onto CM-Sepharose. It exhibited the novel N-terminal sequence, GIEVIKIREL. The deoxyribonuclease was purified to a specific activity of 1584 units/mg. It was devoid of ribonuclease, protease, and HIV-1 reverse transcriptase-inhibitory activities. However, it inhibited cell-free translation in a rabbit reticulocyte lysate system with an IC_{50} of 20 μM. It exhibited antifungal activity toward *Botrytis cinerea* but not toward *Fusarium oxysporum* and *Mycosphaerella arachidicola*.

The seeds of flowering plants constitute a rich source of proteins. A variety of proteins with different functions have been isolated including ribosome inactivating proteins (1–4), lectins (5), ribonucleases (6, 7), amylase inhibitors (8), trypsin and chymotrypsin inhibitors (9, 10), antifungal proteins (11–21), and antimicrobial proteins (22). Many of the aforementioned proteins play a physiological role of defense. They may also possess exploitable activities such as antitumor and antiproliferative (23), immunomodulatory (16, 17), antifungal (11–21), and antiviral enzyme (11, 13) activities.

Asparagus is a popular vegetable in the West as well as in the Orient. Ribosome inactivating proteins designated asparins have been isolated from asparagus (24). We report herein the isolation of a deoxyribonuclease with antifungal activity from asparagus seeds.

MATERIALS AND METHODS

Dried asparagus (*Asparagus officinalis*) seeds were obtained from Harris Moran Seed Co. (CA). The seeds were soaked in saline at 4°C for 2 days owing to the extremely hard nature of the seeds. The seeds were then homogenized. To the saline extract $(NH_4)_2SO_4$ was added to precipitate the proteins. After dialysis the crude proteins were applied to a DEAE–cellulose column (5 × 10 cm) in 10 mM Tris–HCl (pH 7.2). Unadsorbed protein (D1) was removed by elution with the buffer while adsorbed protein (D2) was eluted by addition of 0.5 M NaCl in the buffer. D1 was fractionated by affinity chromatography on Affi-gel Blue gel (2.5 × 20 cm) in 10 mM Tris–HCl (pH 7.2). After elution of unadsorbed protein (B1), adsorbed protein (B2) was eluted by addition of 1.5 M NaCl in 10 mM Tris–HCl (pH 7.2). B1 was next subjected to ion exchange chromatography on a CM-Sepharose column (2.5 × 20 cm) in 20 mM NH_4OAc (pH 4.5). Unadsorbed protein (CM1) was eluted in the buffer. Adsorbed protein was eluted in 3 peaks (CM2, CM3, and CM4) by application of a linear gradient of 0–1 M NaCl in the buffer. The homogeneity as well as the molecular weight of CM3 were assessed by FPLC-gel filtration on Superdex 75. CM3 constituted purified asparagus DNase.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). It was conducted according to the method of Laemmli and Favre (25). After electrophoresis the gel was stained with Coomassie brilliant blue. The molecular weight of asparagus DNase was determined by comparison of its electrophoretic mobility with those of molecular weight marker proteins from Amersham–Pharmacia Biotech.

Amino acid sequence analysis. The N-terminal amino acid sequence of asparagus DNase was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett–Packard 1000A protein sequencer equipped with an HPLC system.

Assay for deoxyribonuclease activity. The reaction mixture consisted of 0.2 ml of 0.1 M NH_4OAc buffer (pH 5.5), 0.2 ml of a herring sperm DNA solution (Gibco BRL) (5 mg/ml) and 10 μl of a asparagus DNase solution (5 mg/ml). Incubation of the reaction mixture was carried out at 25°C for 15 min. The reaction was terminated by addition of 0.35 ml of ice-cold 20 mM lanthanum nitrate in 1.2% perchloric acid. After 20 min at 0°C the reaction mixture was centrifuged at 3000 rpm for 5 min. The supernatant was diluted 3-fold

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with water and the optical density was read at 260 nm against a blank reaction mixture without asparagus DNase. For studying the effect of pH, Mes buffer was used at pH 4, 4.5, 5, and 5.5, NH_4OAc buffer was used at pH 6, 6.5, and 7 and Hepes buffer was used at pH 7.5, 8, 8.5, and 9.5. One unit of enzymatic activity is defined as the amount of enzyme which produces an absorbance increase at 260 nm of 0.001 per minute per milliliter at pH 5.5 and 37°C using herring sperm DNA. It is slightly different from 1 Kunitz unit of DNase activity which is based on a pH of 5, a temperature of 25°C and DNA type 1 as substrate.

Assay of ribonuclease activity. Yeast tRNA was used as substrate asparagus DNase was incubated with 200 μg tRNA in 150 μl 100 mM Hepes buffer (pH 7) before 350 μl ice-cold 3.4% perchloric acid was added to terminate the reaction. After standing on ice for 15 min, the reaction mixture was centrifuged and the absorbance of the supernatant was measured after suitable dilution. One unit of ribonuclease activity is defined as the amount of enzyme which produces an absorbance increase of one per minute in the acid-soluble supernatant per milliliter of reaction mixture under specified conditions (6).

Assay of antifungal activity. The assay for antifungal activity toward *B. cinerea* and other fungal species was carried out in 100 × 15-mm petri plates containing 10 ml of potato dextrose agar. At a distance of 0.5 cm away from the rim of the mycelial colony were placed sterile blank paper disks (0.625 cm in diameter). An aliquot (6 μl) of the asparagus DNase was added to a disk. The plates were incubated at 23°C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity (26).

Assay for protease activity. A solution of casein (Sigma), which was used as the substrate in the protease assay was freshly prepared as follows. To 2 g casein were added 10 ml distilled water and 10 ml 0.2 M NaOH. After addition of another 60 ml distilled water the solution was stirred to make a solution. The pH of the solution was adjusted to 8 with 0.1 M HCl. The volume was made up to 100 ml with distilled water and the solution was heated at 90°C for 15 min before cooling down and dilution with 100 ml 100 mM Tris–HCl (pH 8) containing 40 mM $CaCl_2$. The precipitate was removed and the resulting solution could be used.

Asparagus DNase or trypsin solution (50 μl) was mixed with 350 μl of the above casein solution. After 25 min, 1 ml 5% trichloroacetic acid was added. The reaction mixture was allowed to stand at room temperature for 30 min before centrifugation for 15 min. The absorbance of the supernatant was read at 280 nm against water as blank.

Assay for cell-free translation-inhibitory activity. Assay of cell-free translation inhibiting activity was based on the ability to inhibit protein synthesis from [^{3}H]leucine in a cell-free rabbit reticulocyte lysate system. It has been described previously (3). Many antifungal proteins have been reported to have cell-free translation-inhibitory activity. Asparagus DNase was thus tested for this activity. Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. An assay based on the rabbit reticulocyte lysate system (3) was used. Asparagus DNase (10 μl) was added to 10 μl of hot mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine, and 1 μCi [4,5-^{3}H]leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 μM hemin and 5 μl creatine kinase. Incubation proceeded at 37°C for 30 min before addition of 330 μl 1 M NaOH and 1.2% H_2O_2. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casein hydrolyzate in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on a glass fiber Whatman GF/A filter, washed and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The filter was suspended in scintillant and counted in an LS6500 Beckman liquid scintillation counter.

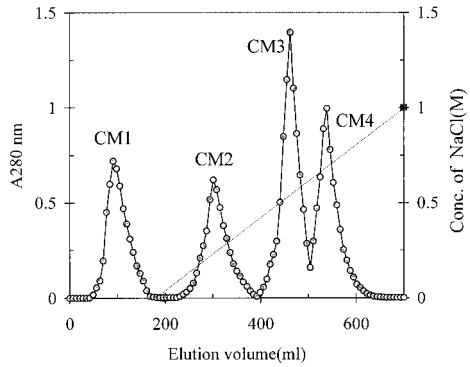

FIG. 1. Ion-exchange chromatography on a CM-Sepharose column (2.5 × 20 cm). Sample: fraction of asparagus seed extract unadsorbed on DEAE–cellulose and Affi-gel blue gel. Buffer: 20 mM NH_4OAc (pH 4.5). The slanting line across the chromatogram represents application of the linear concentration gradient (0–1 M NaCl) to elute adsorbed proteins.

Assay for HIV reverse transcriptase inhibitory activity. Asparagus DNase was tested for this activity since some antifungal proteins possess an inhibitory activity toward HIV-1 reverse transcriptase. The assay for ability to inhibit HIV-1 reverse transcriptase was assessed by using an ELISA kit from Boehringer Mannheim (Germany) as described by Collins *et al.* (27). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(a) · oligo (dT)15. In place of radiolabeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of castamollin was calculated as percent inhibition compared to a control without the protein.

RESULTS

The asparagus seed extract was fractionated on DEAE–cellulose into an unadsorbed fraction D1 with DNase activity and an adsorbed fraction D2 without DNase activity. D1 was separated on Affi-gel blue gel into an unadsorbed fraction B1 with DNase activity and an inactive adsorbed fraction B2. B1 was separated by ion exchange chromatography on CM-Sepharose into an unadsorbed fraction CM1, and three adsorbed fractions CM2, CM3 and CM4 (Fig. 1). Only CM3 exhibited DNase activity. CM3 appeared as a single peak with a molecular weight of 30 kDa in gel filtration on Superdex 75 (data not shown). It also demonstrated a single band with a molecular weight of 30 kDa in SDS–PAGE (Fig. 2). CM3 was obtained with a yield of 527 mg and an activity of 1584 u/mg from

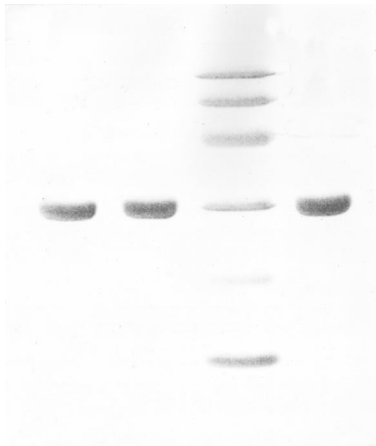

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of asparagus DNase. From left to right: lanes 1, 2, and 4, asparagus DNase; lane 3, molecular weight standards; from top down, phosphorylase *b* (MW 94 kDa), bovine serum albumin (MW 67 kDa), ovalbumin (MW 43 kDa), carbonic anhydrase (MW 30 kDa), trypsin inhibitor (MW 20 kDa), and α-lactalbumin (MW 14.4 kDa).

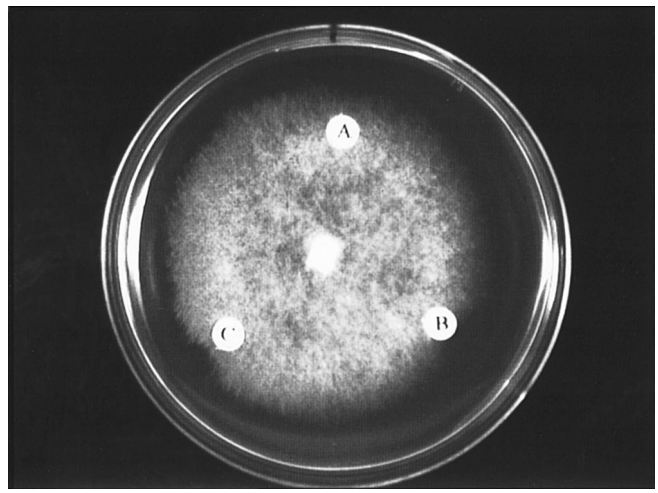

FIG. 4. Antifungal activity of asparagus DNase toward *Botrytis cinerea*. (A) Negative control (0.1 M Mes, pH 6.0). (B) Asparagus DNase (150 μg in Mes buffer). (C) Asparagus DNase (30 μg in Mes buffer).

800 g seeds. The N-terminal sequence of the purified asparagus deoxyribonuclease was GIEVIKIREL.

The optimal pH for the DNase activity of asparagus DNase was 7.5 (Fig. 3). It exerted antifungal activity against *B. cinerea* (Fig. 4), but not against *Fusarium oxysporum*, *Rhizoctonia solani*, and *Mycosphaerella arachidicola*. It inhibited translation in the cell-free rabbit reticulocyte lysate system with an IC_{50} of 20 μM. However, there was no ribonuclease, protease or anti-human immunodeficiency virus type 1 reverse transcriptase activity (Table 1).

DISCUSSION

In this investigation a deoxyribonuclease with antifungal activity toward *B. cinerea* was isolated from asparagus seeds. The DNase possessed very low translation-inhibiting activity (IC_{50} in μM), much lower than that of ribosome inactivating proteins (IC_{50} in nM) (1, 2) but similar to that of antifungal proteins (IC_{50} in μM) (11, 13, 16). Some milk proteins including lactoferrin, lactogenin, glycolactin, and angiogenin exhibit both DNase and translation-inhibitory activities (Wang, Ye, and Ng, unpublished data). Some nucleases demonstrate both deoxyribonucleolytic and ribonucleolytic activities (28), but asparagus DNase lacks RNase activity and its nuclease activity is specific toward DNA. Its DNase activity (1584 units/mg) is more potent than that of DNase I from Sigma (400–800 units/mg) and the milk protein lactoferrin (about 300 units/mg) when compared in the same experiment. The antifungal activity of asparagus DNase exhibits species specificity because *B. cinerea* but not *F. oxysporum* or *M. arachidicola* is sensitive to the inhibitory effect of the protein.

The low cell-free translation-inhibiting activity of asparagus DNase excludes the possibility that aspar-

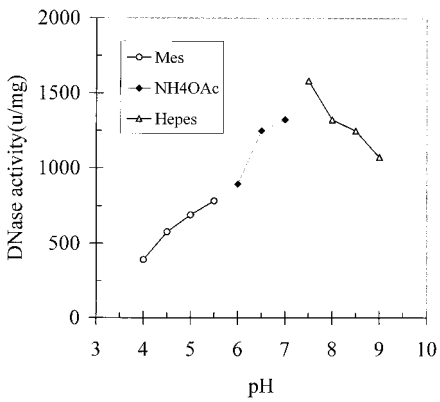

FIG. 3. Determination of optimal pH for asparagus DNase.

TABLE 1

DNase Activity of Various Chromatographic Fractions

Chromatographic fraction	DNase activity (u/mg)
D1	108
D2	6.6
B1	272
B2	5.8
CM1	3.1
CM2	153
CM3	1584
CM4	248

Note. One unit of activity produces a ΔA_{260} of 0.001/min/ml at 25°C.

agus DNase is a ribosome inactivating protein. This is important because asparins, the asparagus ribosome inactivating proteins, have a similar molecular weight (24) and some ribosome inactivating proteins manifest DNase (29) and antifungal (20, 21) activities. The distinctive N-terminal sequence of asparagus DNase also distinguishes it from ribosome inactivating proteins (24). The chromatographic behavior of asparagus DNase on Affi-gel blue gel is also different from that of ribosome inactivating proteins: the former is unadsorbed while the latter are unadsorbed (1, 3).

Asparagus DNase differs from other antifungal proteins (11, 13) and ribosome inactivating proteins (1, 3) chromatographically in that it is unadsorbed on Affi-gel blue gel. However, it is similar to antifungal proteins (25) and ribosome inactivating proteins (1, 3) in that it is unadsorbed on DEAE–cellulose and adsorbed on CM–cellulose, indicating that they are all basic proteins. Unlike other antifungal proteins (13, 25) and ribosome inactivating proteins (30), asparagus DNase does not inhibit HIV-1 reverse transcriptase. Both asparagus DNase and antifungal proteins (11, 13) inhibit translation in the cell-free rabbit reticulocyte lysate system with low potencies. The antifungal activity of asparagus DNase shows some differences from those of antifungal proteins. The latter usually suppress mycelial growth in a number of fungal species (11, 13–16), whereas the former is active against only one of the three fungal species tested.

Proteins with RNase activity including quinqueginsin from American ginseng (31), panaxagin from Chinese ginseng (32) and a heterodimeric protein from *Panax notoginseng* (33) manifest antifungal activity. The milk protein lactoperoxidase, which has a DNase activity of 25 units/mg (Wang and Ng, unpublished data), also displays antifungal activity (34). It is noteworthy that asparagus DNase likewise express an antifungal activity.

Asparagus DNase has a molecular weight of 30 kDa. This molecular weight is close to the molecular weights of most type 1 RIPs (2, 24). Some antifungal proteins also have a molecular weight similar to that of asparagus DNase (11, 13). Aoyagi *et al.* (35) have reported the isolation and characterization of cDNAs encoding a apoptosis-associated DNase, a 35-kDa barley nuclease and a 43-kDa zinnia nuclease.

DNase in seeds plays a physiological role. The amount of water received by seed embryos determines the extent of cell hydration which in turn determines the pattern of degradation of genomic DNA, whether by DNase nucleosome cleavage or by random fragmentation. In accelerated aged and primed seeds water availability dictates progress of the first cell cycle to the first mitotic divisions. The loss of desiccation tolerance at the border of G2M and cytokinesis is related to DNase, DNase conformation, DNA damage and stages of arrest in the first cell cycle (36). Barley nuclease is secreted from the aleurone layer into the endosperm during germination and may be responsible for nuclear DNA digestion during the course of endosperm degradation. Zinnia nuclease appears transiently in the process of differentiation into tracheary elements and may participate in the autolysis at the last differentiation step (35). Most plants have developed mechanisms including DNase to prevent foreign informative molecules from taking over important processes in their cells (37).

All in all, asparagus DNase demonstrates a novel N-terminal sequence, a potent DNase activity and a unique antifungal activity.

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