

## Potyviral NIa Proteinase, a Proteinase with Novel Deoxyribonuclease Activity\*

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**The NIa proteinase from pepper vein banding virus (PVBV) is a sequence-specific proteinase required for processing of viral polyprotein in the cytoplasm. It accumulates in the nucleus of the infected plant cell and forms inclusion bodies. The function of this protein in the nucleus is not clear. The purified recombinant NIa proteinase was active, and the mutation of the catalytic residues His-46, Asp-81, and Cys-151 resulted in complete loss of activity. Most interesting, the PVBV NIa proteinase exhibited previously unidentified activity, namely nonspecific double-stranded DNA degradation. This DNase activity of the NIa proteinase showed an absolute requirement for Mg<sup>2+</sup>. Site-specific mutational analysis showed that of the three catalytic residues, Asp-81 was the crucial residue for DNase activity. Mutation of His-46 and Cys-151 had no effect on the DNase activity, whereas mutant D81N was partially active, and D81G was completely inactive. Based on kinetic analysis and molecular modeling, a metal ion-dependent catalysis similar to that observed in other nonspecific DNases is proposed. Similar results were obtained with glutathione S-transferase-fused PVBV NIa proteinase and tobacco etch virus NIa proteinase, confirming that the DNase function is an intrinsic property of potyviral NIa proteinase. The NIa protein present in the infected plant nuclear extract also showed the proteinase and the DNase activities, suggesting that the PVBV NIa protein that accumulates in the nucleus late in the infection cycle might serve to degrade the host DNA. Thus the dual function of the NIa proteinase could play an important role in the life cycle of the virus.**

The genus *Potyvirus* is the largest plant virus group comprising more than 30% of all known plant viruses and is economically very important (1). The name potyvirus is derived from the type member of the group, the potato Y virus. It belongs to the Potyviridae family, which includes five other genera. All members of the genera in this family have similar genome organization and gene expression strategies and are grouped under the “Picorna-like superfamily” of viruses (2). The virions of potyviruses are flexuous rod-shaped particles of 700–900 nm in length and 9–11 nm width. The genome is a single-stranded positive sense RNA of about 10 kb length, which is encapsidated by ~2000 identical coat protein subunits of 30–37 kDa (1). Pepper vein banding

virus (PVBV)<sup>1</sup> is the most prevalent virus infecting chili pepper in India. Recently, the complete genomic sequence of the virus was determined, and phylogenetic analysis clearly established that PVBV is a distinct member of the *Potyvirus* genus (3). The PVBV genome is 9771 nucleotides in length with 5'- and 3'-nontranslated end regions and a poly(A) tail at the 3'-end. As in other potyviruses, the PVBV genome contains a single large open reading frame, which can be translated to a polyprotein of 340 kDa that undergoes proteolytic processing predominantly by a viral proteinase, called nuclear inclusion proteinase-a (NIa proteinase) (4).

The potyviral NIa proteinases share structural motifs typical of cellular serine proteinases, although there is no sequence similarity, with a Cys rather than Ser at the active site (5–7). Site-specific mutagenesis studies with tobacco etch virus (TEV) (13), Plum pox potyvirus (8), and PVBV NIa proteinases (9) have unequivocally established the role of His-Asp-Cys in forming the catalytic triad responsible for the cleavage by the proteinase. The specificity of these proteinases is determined by the heptapeptide recognition sequence surrounding each cleavage site (10, 11). NIa proteinase cleaves the polypeptide on the C-terminal side of a Gln or Glu residue at the P1 position within the heptapeptide sequence. Most interesting, another serine-like cysteine protease, picornaviral 3C protease, also has similar specificity of cleavage. Recently, the structure of TEV NIa proteinase complexed with a substrate peptide was determined (12). The structure of the NIa proteinase was found to be similar to that of picornavirus 3C protease (12). The NIa protein has two domains, the C-terminal NIa-proteinase domain and the N-terminal viral genome-linked protein domain (13). As the name suggests, it forms “inclusion bodies” inside the nucleus of infected cells at a late stage of infection (2, 14). NIa protein has independent nuclear localization signals that allow it to accumulate in the nucleus, although their function in the nucleus is unclear (15–17). The NIa proteinase was also shown to bind to RNA nonspecifically, suggesting its involvement in virus replication (18). Thus the NIa functions as a multifunctional protein in the infected cell cytoplasm.

In the present investigation, the wild-type and the site-specific mutants of PVBV NIa proteinase were used to establish an additional enzymatic activity of NIa proteinase, *i.e.* nonspecific dsDNA degradation. Furthermore, the NIa protein in the nucleus of infected leaves was shown to be a dual function enzyme, containing both the proteinase and the DNase activities. It is possible that the NIa proteinase with this novel

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<sup>1</sup> The abbreviations used are: PVBV, pepper vein banding virus; TEV, tobacco etch virus; GST, glutathione S-transferase; GFP, green fluorescence protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; Ni-NTA, nickel-nitrilotriacetic acid; MES, morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

DNase activity might play an important role in host cell DNA degradation in the later stages of infection cycle.

#### EXPERIMENTAL PROCEDURES

**Materials**—Restriction endonucleases and modification enzymes were obtained from MBI Fermentas. Deep Vent DNA polymerase and DpnI were obtained from New England Biolabs. Ampicillin, IPTG, Coomassie Brilliant Blue R-250, acrylamide, EDTA, imidazole, reduced glutathione, Agarose, Triton-X100, 2-mercaptoethanol,  $MgCl_2$ , and all the buffers used were obtained from U. S. Biochemical Corp. Glutathione-Sepharose and Ni-NTA-agarose were obtained from Amersham Biosciences and Qiagen, respectively. The oligonucleotide primers were purchased from Sigma.  $\lambda$  phage DNA, M13 single-stranded DNA, was obtained from Bangalore Genei, India. All other chemicals were of analytical grade.

**Construction of Expression Plasmids and Point Mutants in *Escherichia coli***—The cloning of the NIa gene in pRSET-C (Invitrogen) and the construction of pRNiA and site-specific mutant pRNiA C151A and pRNiA D81N clones have been described previously (9). Using pRNiA as template and the corresponding set of mutant primers (H46-sen, 5'-CAA ATC GCG CTT TAC TC-3', His-46-anti, 5'-GAG TAA AGC GCG ATT TG-3', D81G-sen, 5'-GTA GAG AAA CAT GGA ATT CTG CTC-3', and D81G-anti, 5'-GAG CAG AAT TCC ATG TTT CTC TAC-3'), the mutant clones pRNiA-H46A and pRNiA-D81G were constructed by the PCR-based method (19). The clones pRNiA, pRNiA H46A, pRNiA D81N, pRNiA D81G, and pRNiA C151A were used for the expression of His-tagged NIa proteinase and its mutants as described earlier (9).

To obtain GST-fused NIa proteinase and site-specific mutants, NIa and the mutants were PCR-amplified with a pair of specific primers (9) using pRNiA and the corresponding mutants as template and subcloned at a SmaI site of pGEX-5X-2 (Amersham Biosciences), and the resulting constructs were named pGNiA, pGNiA-D81G, pGNiA D81N, pGNiA H46A, and pGNiA C151A. All the plasmids were sequenced to confirm the presence of mutations.

**Expression and Purification of Fusion Proteins**—*E. coli* BL21 (DE3) cells transformed with pRNiA or pGNiA or mutant constructs were grown and induced for protein expression. A single colony was inoculated in 50 ml of Luria-Bertani (LB) medium containing 100  $\mu$ g/ml ampicillin and grown overnight at 30 °C. This overnight culture was inoculated into 0.5 liters of LB medium containing 100  $\mu$ g/ml ampicillin. After 4 h of growth at 30 °C, the cells were induced with 0.3 mM (final concentration) isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 4 h, and the cells were harvested by centrifugation. The cells from 1 ml of culture were used for checking the expression by SDS-PAGE analysis on 12% gel (20). Proteins were stained with Coomassie Brilliant Blue or immunoblotted (21) with rabbit polyclonal antiserum raised against purified His-tagged NIa proteinase (22) to confirm their identity. Purification of His-tagged proteins was carried out as described previously (9). Purification of GST-NiA proteinases was carried out using glutathione-Sepharose 4B as described by the manufacturers. Typical yields after elution with glutathione were 5 mg of protein/liter of culture. We obtained the plasmid pTEV-NiA as a kind gift from Dr. Michael Ehrmann. The recombinant His-tagged TEV NiA proteinase was purified similarly as described for PVBV NiA proteinase. The PVBV NiA proteinase and the mutants were further purified by gel filtration on Sephacryl-S200 (Amersham Biosciences) column (90  $\times$  1 cm, 60 ml). The column was equilibrated with 25 mM Tris-HCl buffer, pH 8, and calibrated using gel filtration standard (Sigma) for size exclusion chromatography. Purity of the proteins was checked by SDS-PAGE (20) and MALDI-MS (Amersham Biosciences).

**Construction, Expression, and Purification of the GFP Substrate**—Green fluorescence protein (GFP) coding region was PCR-amplified using a pair of specific primers, GFP-sen, (5' CGG TGA AGT TGC/CCA TCA GGC AGG AGA ATG GTG AGC AAG GGC GAG GAG CTG TTC-3') GFP-anti (5'-CTT GTA CAG CTC GTC CAT GCC GAG AGT-3') from the vector pProEX-HT (Invitrogen) and cloned at PvuII site of pRSET-C. The underlined nucleotides in GFP-sen primer represent nine amino acid residues corresponding to the conserved cleavage recognition sequences of PVBV NiA proteinase (NH<sub>2</sub>-GEVAHQ↓AGE-COOH). The expressed protein from the recombinant clone (pSubGFP) would have 41 residues (including 6 histidines) from the vector and 9 residues corresponding to the cleavage recognition sequence of PVBV NiA proteinase at the N terminus of GFP. Such a fused protein can serve as substrate for NIa proteinase and was therefore called GFP substrate. *E. coli* BL21 (DE3) cells transformed with pSub-GFP were grown in the presence of 100  $\mu$ g/ml ampicillin and induced with IPTG (0.3 mM) for overexpression. Purification of GFP substrate fusion pro-

tein was carried out using Ni-NTA-agarose affinity chromatography as described by the manufacturer (Qiagen). Typical yields after elution with imidazole were 15 mg protein/liter culture. Purity of the protein was monitored by SDS-PAGE, and the presence of the cleavage site residues was confirmed by PVBV NiA proteinase assay. NIa proteinase assay with GFP substrate in solution was carried out as follows. GFP substrate (100  $\mu$ g) was incubated with 1  $\mu$ g of His-tagged or GST-fused PVBV NiA proteinase in 50  $\mu$ l of assay buffer (200 mM NaCl, 25 mM Tris-HCl, pH 8.2, and 10% glycerol) at 37 °C for 4 h. For assay with the plant nuclear extract, total extract was taken as a source of proteinase at a final concentration of 100  $\mu$ g in 50  $\mu$ l of assay buffer and incubated at 37 °C for 4 h. The cleavage by the NIa proteinase was monitored by SDS-PAGE followed by Western analysis using anti-GFP rabbit polyclonal antiserum raised against Ni-NTA affinity-purified GFP obtained from expression of pRGFP.

**NIa Proteinase Assay with Bead-attached GFP Substrate**—*In vitro* bead-attached GFP substrate cleavage by NIa proteinase was carried out as described by Patel *et al.* (23) with some modification. Briefly, 25 ml of *E. coli* BL21 (DE3) cells harboring pSub-GFP were grown and induced for expression. The cell pellet was resuspended in 5 ml of binding buffer (25 mM Tris-HCl, 200 mM NaCl, pH 8.2) and lysed by mild sonication. Ni-NTA-agarose slurry (200  $\mu$ l) was added to the supernatant obtained after centrifugation of cell lysate and incubated for 30 min. GFP substrate-bound Ni-NTA-agarose beads were washed three times with wash buffer (binding buffer with 30 mM imidazole) followed by a final wash with binding buffer. Parallel experiments were carried out to determine the amount of bound GFP substrate by eluting the protein from the washed beads by adding 300 mM imidazole. Substrate-bound beads were resuspended in reaction buffer (binding buffer with 10% glycerol), and the final concentration of the substrate was adjusted to 0.5 mg/ml. His-tagged PVBV NiA proteinase was added at a final concentration of 50  $\mu$ g/ml, and the proteinase reaction mixture was incubated with intermittent gentle agitation at 37 °C for 60 min. Agarose beads attached to uncleaved substrate were removed by brief centrifugation. The supernatant retained the cleaved product (GFP) and the amount of GFP released was measured by GFP fluorescence (excitation at 490 nm and emission at 510 nm) using a spectrofluorimeter (PerkinElmer Life Sciences) over the blank reaction where no enzyme was added. A standard curve was generated by plotting the relative fluorescence emission of recombinant GFP at 510 nm as a function of GFP concentration and was used as reference for determining the amount of product formed by the PVBV NiA proteinase.

**DNA Binding Analysis**—A 300-bp PCR product (4  $\mu$ g),  $\lambda$  phage DNA (0.5  $\mu$ g), or M13 single-stranded DNA (0.5  $\mu$ g) was incubated with a range of concentrations (0.5–2  $\mu$ g) of PVBV His-tagged NIa proteinase in 20 mM Tris-HCl, pH 8.2, 0.5 mM EDTA at 37 °C for 0.5 h in a final volume 40  $\mu$ l. After stopping the reaction by addition of 6  $\mu$ l of loading dye, 10  $\mu$ l of each sample was loaded in 0.7% agarose gel and electrophoresed in 1 $\times$  TAE. The DNA was visualized by ethidium bromide staining.

**Deoxyribonuclease Activity**—DNase reaction mixture, containing  $\lambda$  phage DNA or M13 single-stranded DNA or double-stranded pUC-19 vector DNA (5  $\mu$ g) and purified recombinant His-tagged or GST-NiA proteinase (1  $\mu$ g), was incubated at 37 °C for 45 min in 20 mM Tris-HCl, pH 8.2, and 1 mM  $MgCl_2$  in a final volume 40  $\mu$ l. For each reaction sample, 6  $\mu$ l of DNA loading dye (MBI Fermentas) was added to terminate the reaction. 5  $\mu$ l of each sample was loaded in 0.7% agarose gel and electrophoresed in 1 $\times$  TAE, and the DNA was visualized by ethidium bromide staining. In the reactions involving total nuclear extract, 5  $\mu$ g of total nuclear protein was used instead of NIa proteinase. DNA was visualized under ultraviolet light by ethidium bromide staining. DNase activity was also estimated quantitatively by measuring the liberated acid-soluble oligonucleotides and mononucleotides as described by Rashid *et al.* (24) with some modifications. The reaction mixture contained 50  $\mu$ g of  $\lambda$  phage DNA, 5  $\mu$ g of purified His-tagged or GST-NiA proteinase in 20 mM Tris-HCl, pH 8.2, and 1 mM  $MgCl_2$  in a total volume of 60  $\mu$ l. After incubation at 37 °C for 45 min, the reaction was stopped by addition of equal volumes of acid lanthanum reagent (20 mM La(NO<sub>3</sub>)<sub>3</sub> in 0.2 N HCl). The precipitate was removed by centrifugation, and absorbance of the supernatant solution was measured at 260 nm in a spectrophotometer (Shimadzu, Japan) against a blank sample without enzyme. The amount of product released was calculated by using an average molar absorption coefficient of 10,750 per nucleotide at 260 nm (25). Plots of nanomoles of product released per min/mg of protein *versus* DNA concentration were hyperbolic for wild-type and the mutant NIa proteinases, and the data were fit to the Michaelis-Menten equation to generate apparent  $V_{max}$  and  $K_m$  values. Although the present study could not provide absolute steady state values be-

cause of polymeric nature of the substrate, useful information was obtained by a comparison of  $V_{\max}$  values between the mutants and the wild-type enzyme. The  $k_{\text{cat}}$  values for these enzymes were calculated from the respective  $V_{\max}$  values using a molecular mass of 33,524.2 Da for the enzymes (25).

**Preparation of Nuclei from the Plant Tissue**—Nuclei from healthy and infected plants were prepared by a method described by Cheah and Osborne (26) with minor modifications. Minced leaves were homogenized in a pre-chilled mortar and pestle in homogenization buffer (0.3 M sucrose in 10 mM KCl, 10 mM NaCl, 15 mM  $\beta$ -mercaptoethanol, 15 mM Tris-HCl, pH 7.4). The homogenate was filtered through three layers of Miracloth (Calbiochem). The residue was washed with homogenization buffer containing 1% Triton X-100 and was pelleted at  $350 \times g$  for 8 min in a centrifuge (Hitachi). The pellet was resuspended in homogenization buffer without Triton X-100 and centrifuged as before. The final pellet was resuspended in resuspension buffer (homogenization buffer containing 20% v/v glycerol) and stored at  $-70^\circ\text{C}$ . All the procedures were carried out at  $0-4^\circ\text{C}$ .

Nuclear extract was prepared as described by Parker and Topol (27) with some modification. Frozen nuclei were thawed, and an equal volume of homogenization buffer was added. Nuclei were pelleted by centrifugation as before and dissolved in nuclear lysis buffer (150 mM KCl, 1 mM dithiothreitol, in 25 mM Tris-HCl, pH 7.5, containing 1 mM  $\text{MgCl}_2$ ) with gentle stirring. Total protein was precipitated by 80% ammonium sulfate, and the pellet was dissolved in nuclear extraction buffer (NEB: 25 mM KCl, 1 mM dithiothreitol, in 25 mM Tris-HCl, pH 7.5, containing 10% glycerol) and dialyzed overnight against NEB. Total protein in the extract was determined with the Bio-Rad Protein Assay System using bovine serum albumin as a standard.

**Immunodepletion Experiments**—For immunodepletion, total nuclear protein concentration was adjusted to 2 mg/ml. Any unsolubilized material was discarded by centrifuging at  $12,000 \times g$  for 30 min at  $4^\circ\text{C}$ . 400  $\mu\text{l}$  of the supernatant was incubated for 2 h with 100  $\mu\text{l}$  of 0.1 M Tris-HCl buffer, pH 7.5, containing 10  $\mu\text{g}$  of affinity-purified rabbit polyclonal anti-NIa antibodies or nonspecific rabbit IgGs (as a negative control). Subsequently, 100  $\mu\text{l}$  of pre-washed protein-A-Sepharose was added to the sample and incubated for 1 h at  $4^\circ\text{C}$ . Immunodepleted supernatants were separated from the pellets by centrifugation at  $12,000 \times g$  for 30 s.

## RESULTS

**Expression and Purification of PVBV NIa Proteinase and Its Mutants in *E. coli***—PVBV NIa proteinase and its mutants were overexpressed in *E. coli* as N-terminal histidine tag or GST-fused proteins. SDS-PAGE analysis of IPTG-induced *E. coli* showed the induction of 33.5-kDa His-tagged (Fig. 1A, lane 2) and 58-kDa GST fusion NIa proteinase (Fig. 1C, lane 2). The His-tagged proteinase was purified by Ni-NTA affinity chromatography (Fig. 1B, lane 1), whereas GST fusion protein was purified using glutathione-Sepharose affinity chromatography (Fig. 1D, lane 1). The affinity-purified proteins were further purified by gel filtration chromatography, and their native molecular weight was determined (Fig. 1E). The His-tagged NIa proteinase eluted as a monomer at 28.6 ml corresponding to 34 kDa (solid line, Fig. 1E), whereas the GST-fused NIa proteinase eluted at 24.1 ml as a dimer of 110-kDa size (dotted line, Fig. 1E). The dimer formation could be because of GST, as GST is known to exist as dimers (28), and in this instance a monomer (NIa) has “dimerized” as a GST fusion protein. The purity of the NIa proteinase was assessed by MALDI-MS, and the molecular weight of His-tagged NIa proteinase was determined to be 33,456.3 Da (Fig. 1F), which was in agreement with the expected  $M_r$  of 33524.2. Two of the catalytic triad residues of PVBV NIa proteinase Asp-81 and Cys-151 were mutated to Asn and Ala, respectively, and were shown to be inactive (9). In the present study His-46 was mutated to Ala and Asp-81 was mutated to Gly as described under “Experimental Procedures.” These mutants, H46A, C151A, D81N, and D81G, were expressed both as N-terminal His tag or GST fusion proteins and purified by Ni-NTA chromatography or GSH-Sepharose affinity chromatography, respectively, as described earlier. The pu-

urity of the His-tagged H46A, C151A, D81N, and D81G mutant NIa proteinases (Fig. 1B, lanes 2–5) and the corresponding GST fusion proteins (Fig. 1D, lanes 2–5) was checked by SDS-PAGE. These highly pure wild-type and mutant proteins were used for the characterization of the enzymatic properties. His-tagged TEV NIa proteinase was expressed and purified similarly like that of His-tagged PVBV NIa proteinase.

**Characterization of Proteinase Activity**—The proteinase activity of PVBV NIa proteinase and its mutants was evaluated using a new fluorometric procedure. The substrate for the assay was generated as described under “Experimental Procedures.” The protein substrate had three different modules (Fig. 2A), namely binding module (hexahistidine tag), proteinase cleavage module representing nine amino acid recognition sequence ( $\text{NH}_2\text{-GEVAHQ} \downarrow \text{AGE-COOH}$ ), and detection module (green fluorescence protein). If such a protein substrate (37 kDa) were incubated with the purified NIa proteinase, then specific cleavage by the enzyme would result in the release of GFP (31 kDa) that can be monitored by Western analysis by using anti-GFP antibodies. The reaction can also be monitored by using Ni-NTA-agarose bound substrate in which case the GFP released into the buffer is estimated by measuring the relative fluorescence at 510 nm after the excitation at 490 nm. The quantity of the released GFP can be estimated from the standard curve of relative fluorescence as a function of GFP concentration. As shown in Fig. 2B, the assay with bead-attached substrate resulted in the release of GFP as a function of time, with wild-type His-tagged NIa proteinase, whereas the H46A, D81G, D81N, and C151A mutant NIa proteinases did not show any substrate cleavage activity.

The progression of the cleavage reaction was also monitored by Western analysis using anti-GFP polyclonal antiserum (Fig. 2, C–E). There was a progressive increase in the formation of a 31-kDa GFP with an increase in the time of incubation, and more than 95% of the substrate (100  $\mu\text{g}$ ) (Fig. 2C, lane 9) was cleaved in 90 min by the His-tagged NIa proteinase (5  $\mu\text{g}$ ). These results demonstrate that the solution assay is more efficient than the cleavage of the bead-attached substrate. The efficiency of proteinase reaction was neither affected by addition of divalent cations like  $\text{Mn}^{2+}$  and  $\text{Mg}^{2+}$  nor by the presence of metal chelator EDTA (data not shown). Furthermore, as shown in Fig. 2D, His-tagged H46A, D81G, D81N, and C151A mutant NIa proteinases failed to cleave the protein substrate, and no GFP release (31-kDa band) was observed. GST-fused NIa proteinase also cleaved the GFP substrate (Fig. 2E, lane 2), and as expected, the GST-fused C151A, D81N, H46A, and D81G mutant proteins did not show any release of GFP (Fig. 2E, lanes 3–6, respectively). These results demonstrate that both the recombinant His-tagged NIa proteinase and GST-fused NIa proteinase are active, and His-46, Asp-81, and Cys-151 constitute the catalytic triad in the enzyme. Mutation of any of these residues completely abolished the activity of both the monomeric (His-tagged NIa proteinase) and the dimeric forms (GST-tagged NIa proteinase) of PVBV NIa proteinase.

**DNase Activity of PVBV NIa Proteinases**—Earlier, potyviral NIa proteinase was shown to bind to RNA nonspecifically. Most interesting, when the purified His-tagged PVBV NIa proteinase was tested for its nucleic acid binding properties, we observed that it also binds to single-stranded and double-stranded DNA nonspecifically. As shown in Fig. 3A, His-tagged NIa proteinase could gel-shift a 300-bp PCR-amplified dsDNA fragment, when incubated along with 0.5 mM EDTA. The protein-DNA complex migrated slower than the free DNA, and the large protein-DNA complex remained within the well (Fig. 3A, lanes 3 and 4). Similar results were obtained when double-stranded  $\lambda$  phage DNA (Fig. 3B, lanes 3 and 4) and single-

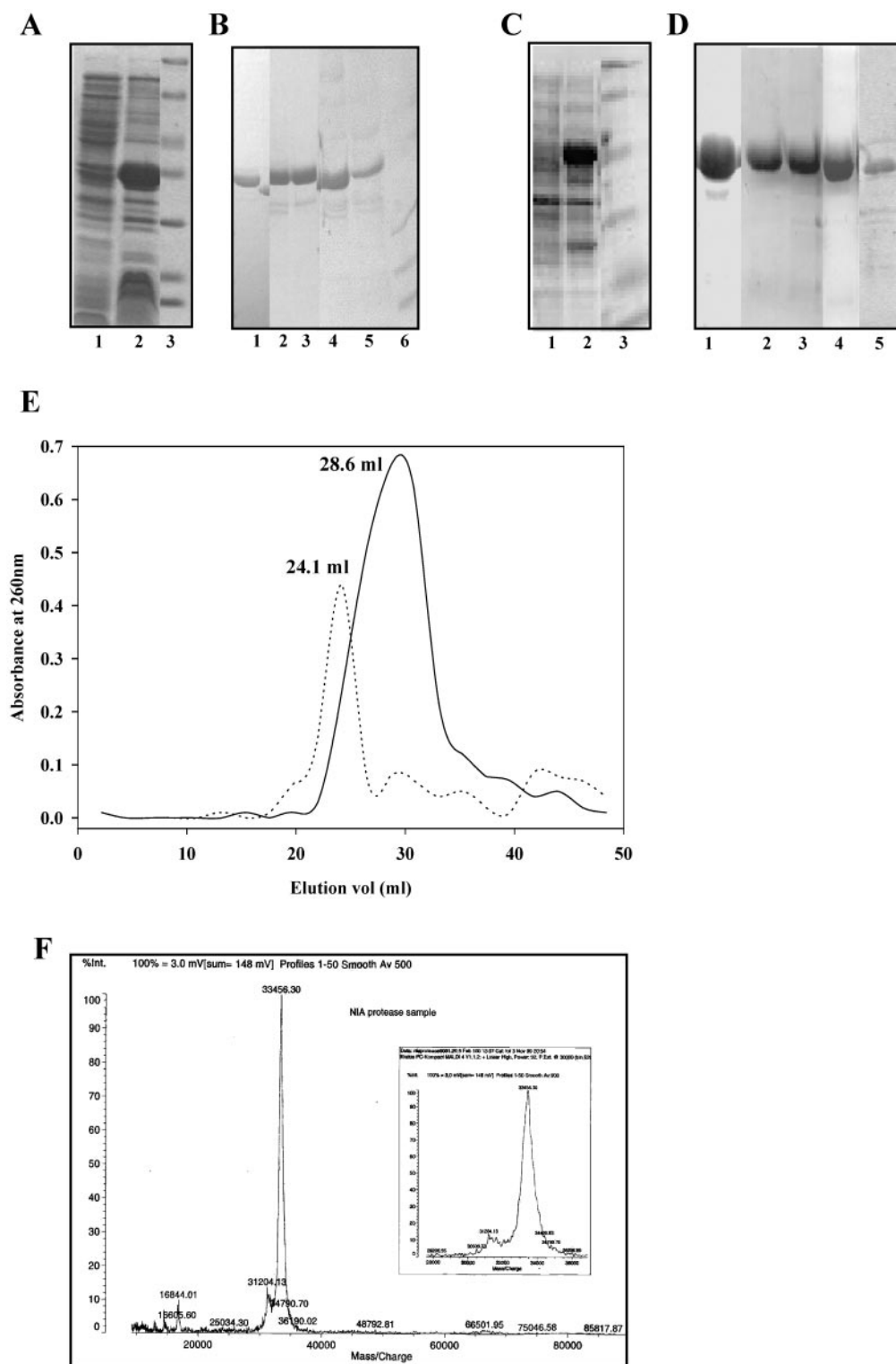
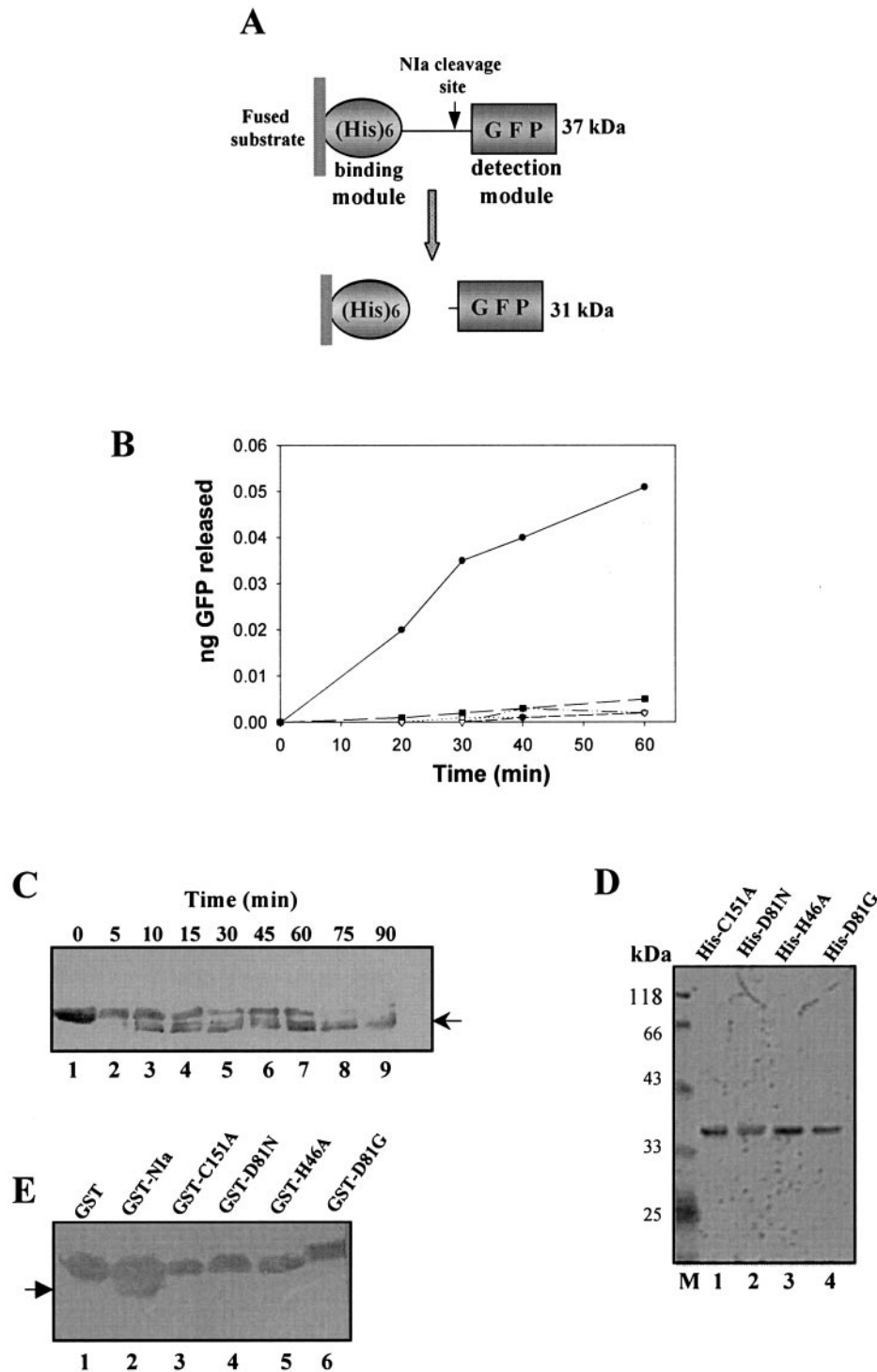


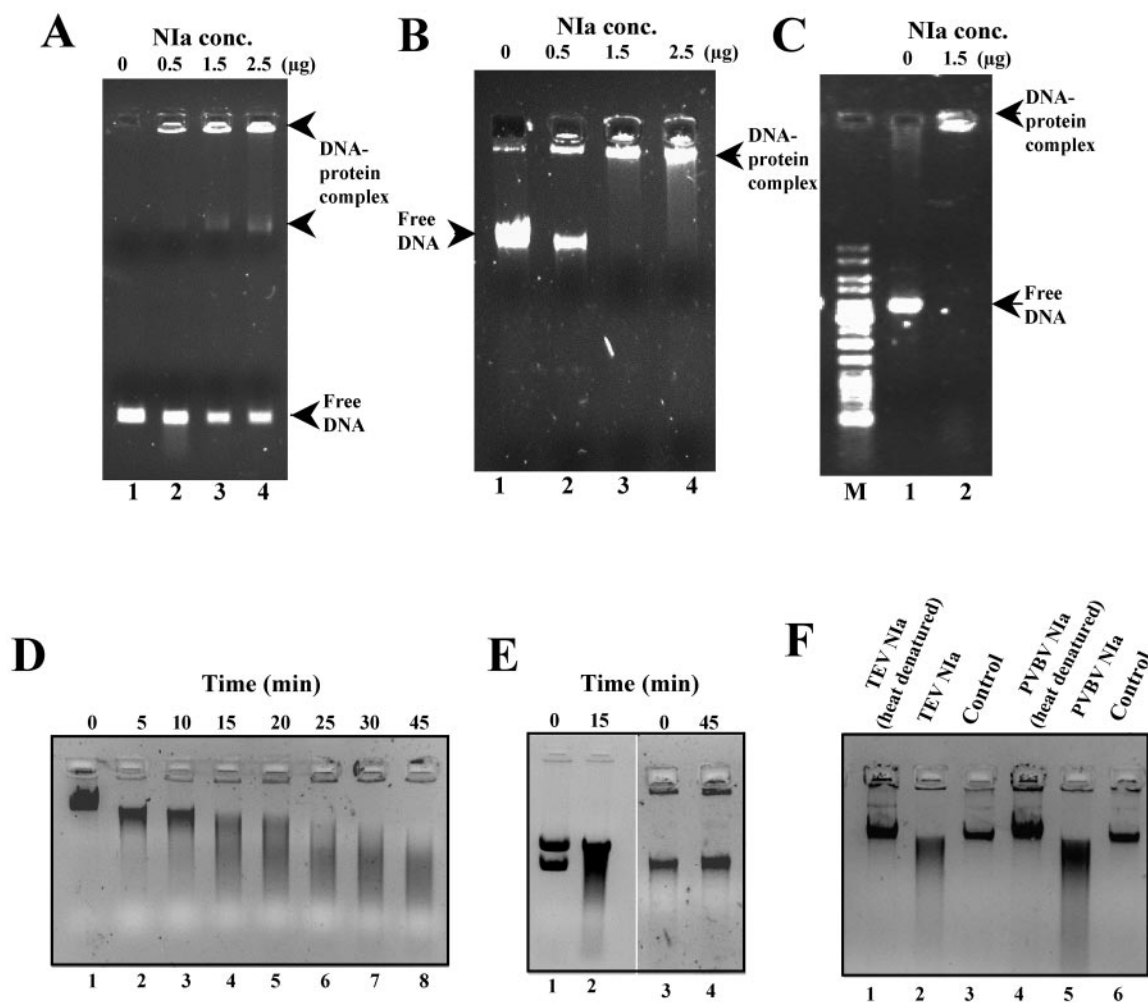
FIG. 1. 12% SDS-PAGE showing expression and purification of recombinant PVBV NIa proteinase and its mutants. *A*, lane 1, total protein from uninduced BL21 (DE3) cells harboring pRNiA; lane 2, total protein after induction with 0.3 mM IPTG for 4 h at 30 °C; lane 3, molecular weight markers. *B*, lanes 1–5 show single step Ni-NTA affinity-purified wild-type His-tagged NIa proteinase, H46A, D81N, D81G, and C151A mutants, respectively. Lane 6 represents molecular wild type markers. *C*, lane 1, total protein from uninduced BL21 (DE3) cells harboring pGNiA; lane 2, total protein after induction. Lane 3 represents molecular weight markers. *D*, lanes 1–5 show glutathione-Sepharose affinity-purified wild-type GST-NiA proteinase, H46A, D81N, C151A, and D81G mutant GST-NiA proteinase, respectively. *E*, Sephacryl S-200 (Amersham Biosciences) gel filtration chromatography (90 × 1 cm, 60 ml) of His-tagged and GST fusion NIa proteinase. Elution profile of GST-fused wild-type NIa proteinase is shown (solid line). The protein eluted as dimer at 24.1 ml. Elution profile of His-tagged wild-type NIa proteinase is shown (broken line). The protein eluted as monomer at 28.6 ml. *F*, MALDI-TOF analysis of His-tagged wild-type NIa proteinase (also inset).

stranded M13 phage DNA (Fig. 3C, lane 3) were incubated with His-tagged NIa proteinase. However, when double-stranded  $\lambda$  DNA was incubated with NIa proteinase at 37 °C in the reac-

tion buffer (20 mM Tris-HCl, pH 8.2, and 1 mM MgCl<sub>2</sub>) without EDTA, there was a linear increase in the degradation of DNA with an increase in the time of incubation, and almost complete



**FIG. 2. Proteinase activity of recombinant PVBV NIa and its mutants.** *A*, schematic representation of bead attached GFP substrate and the cleavage by NIa proteinase. *B*, time course of proteolytic cleavage by NIa proteinase and its mutants as monitored by bead-attached substrate assay. Bead-attached GFP substrate (0.5 mg/ml) was added to His-tagged NIa proteinase and its mutants (50  $\mu$ g/ml), and the reaction mixture was incubated in 25 mM Tris-HCl, 200 mM NaCl, pH 8.2, at 37 °C for 60 min. Uncleaved substrate attached to the agarose bead was removed by brief centrifugation, and GFP released was recovered from the supernatant. Aliquots were taken at different time points, and the product formed was measured by GFP fluorescence ( $A_{\max} = 490$  nm;  $E_{\max} = 510$  nm). WtNIa, D81N NIa, D81G NIa, H46A NIa, and C151A NIa are shown as ●, ∇, ■, and ○, respectively. A standard curve generated by plotting the relative fluorescence of recombinant GFP as a function of protein concentration was used as the reference. *C*, Western analysis of proteolytic degradation of GFP substrate. GFP substrate (100  $\mu$ g) was incubated with 1  $\mu$ g of His-tagged NIa proteinase in 25 mM Tris-HCl, pH 8.2, containing 200 mM NaCl and 10% glycerol (50  $\mu$ l) at 37 °C. Reaction products were analyzed by Western blotting using anti-GFP rabbit polyclonal antiserum after separation on 12% SDS-PAGE. Lane 1 shows intact substrate with no enzyme, and lanes 2–9 represent extent of cleavage at 5, 10, 15, 20, 25, 30, 45, 60, 75, and 90 min, respectively. Arrow indicates the position of the cleaved product. *D*, Western analysis of proteolytic cleavage by mutant His-tagged NIa proteinases. The reaction was carried out for 4 h as described in the legends for *C*. Lanes 1–4 represent reaction products obtained with His-tagged C151A, D81N, H46A, and D81G NIa proteinase mutants as enzyme, respectively. No cleavage of GFP substrate was observed. *M* denotes molecular weight marker. *E*, Western analysis of proteolytic cleavage by GST fused NIa proteinase and its mutants. Lane 1, GST added to GFP substrate, and as expected no cleavage is seen; lane 2, GST-NIa proteinase added to GFP substrate; lanes 3–6, denote reaction products obtained with GST-fused C151A, D81N, H46A, and D81G NIa proteinase, respectively. Arrow indicates the position of the cleaved product.

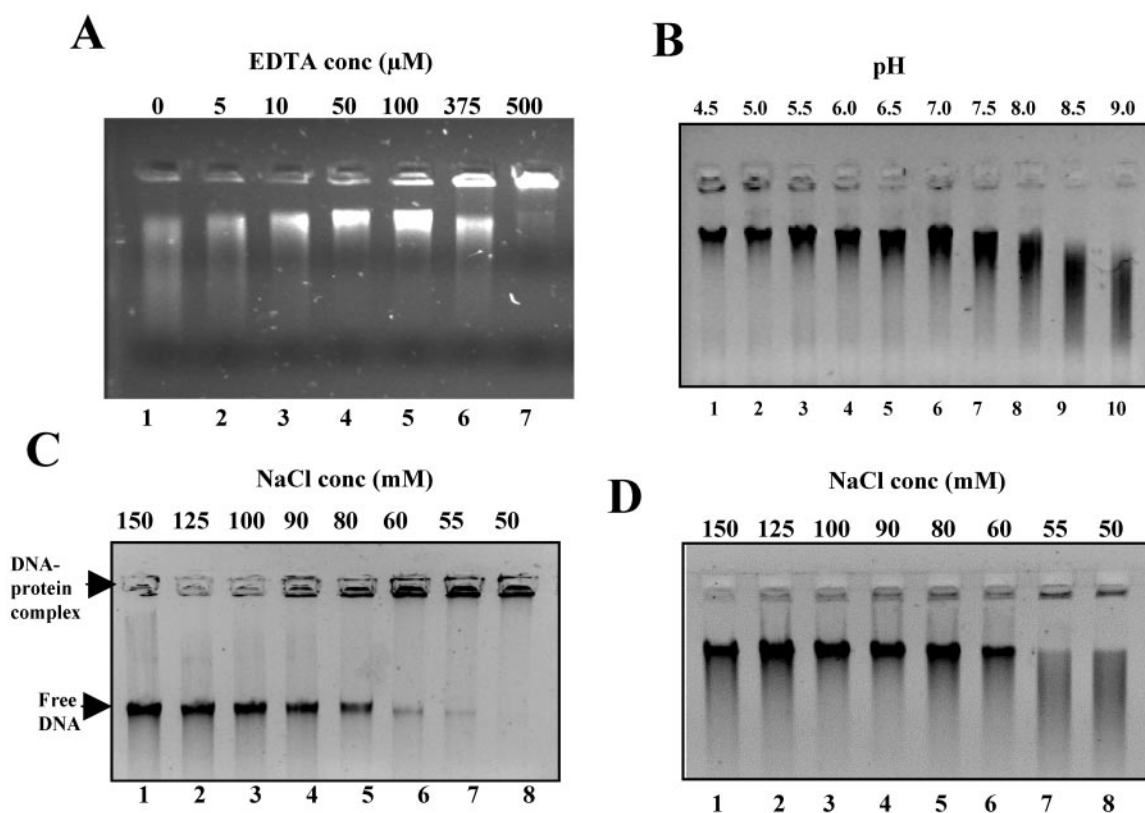


**FIG. 3. Gel shift assay of DNA binding to NIa proteinase.** A, the mobility shift caused by increasing concentrations of PVBV NIa proteinase (0.5–2  $\mu\text{g}$ ) incubated with a 300-bp PCR product (4  $\mu\text{g}$ ) or  $\lambda$  phage DNA (0.5  $\mu\text{g}$ ) (B) or M13 ssDNA (0.5  $\mu\text{g}$ ) (C) in 20 mM Tris-HCl, pH 8.2, 0.5 mM EDTA at 37  $^{\circ}\text{C}$  for 30 min. Samples were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining. Arrowheads indicate the mobility of the free DNA and the DNA-protein complex. Analysis of DNase activity of NIa proteinase is shown. D, substrate  $\lambda$  phage DNA (5  $\mu\text{g}$ ) (lanes 3 and 4) as substrate. Plasmid DNA and single-stranded DNA were incubated for 15 and 45 min, respectively, in the presence of 1 mM  $\text{MgCl}_2$  and were analyzed as described in D. E, DNase activity of PVBV NIa with pUC-19 plasmid DNA (5  $\mu\text{g}$ ) (lanes 1 and 2) and M13 single-stranded DNA (5  $\mu\text{g}$ ) (lanes 3 and 4) as substrate. Plasmid DNA and single-stranded DNA were incubated for 15 and 45 min, respectively, in the presence of 1 mM  $\text{MgCl}_2$  and were analyzed as described in D. F, DNase activity of TEV NIa proteinase. His tag-purified TEV NIa proteinase (1  $\mu\text{g}$ ) was incubated with  $\lambda$  phage DNA (5  $\mu\text{g}$ ) as described in D and analyzed after 15 min (lane 2). When the TEV NIa proteinase was inactivated by heating at 85  $^{\circ}\text{C}$  for 10 min, the denatured enzyme showed no residual activity (lane 1), and the incubation with eluate from extracts of cells harboring control vectors also showed no activity (lane 3). Lanes 4–6 represent reactions carried out with PVBV NIa proteinase under identical conditions.

degradation was observed in 45 min as analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig. 3D, lanes 1–8). NIa proteinase could also cleave pUC-19 plasmid DNA (Fig. 3E, lanes 1 and 2). Most interesting, when single-stranded DNA instead of double-stranded DNA was incubated with PVBV NIa proteinase at 37  $^{\circ}\text{C}$ , no such degradation was observed (Fig. 3E, lanes 3 and 4). We have also tested TEV NIa proteinase, another potyvirus with 48% sequence identity to PVBV NIa proteinase, for such activity. As shown in Fig. 3F, TEV NIa proteinase also showed comparable DNase activity as that of PVBV NIa proteinase under similar experimental conditions (lanes 2 and 4). In order to confirm that the observed DNase activity is not from any undetectable contaminant from the host cells, comparable amounts of host cells with vectors were taken through the same purification protocol. Such samples did not show either DNase binding or DNase activity at 37  $^{\circ}\text{C}$  (Fig. 3F, lanes 3 and 6). Furthermore, the observed dsDNA degradation is not due to nonenzymatic cleavage as control experiments with heat-denatured enzyme did not show any degradation (Fig. 3F, lanes 1 and 4). The reaction is metal

ion-dependent as no degradation was observed in the presence of EDTA (Fig. 3B).

Examination of the catalytic site of various DNases, such as DNase I, DNA polymerase I 3'–5' exonuclease, nuclease P1, and restriction enzymes EcoRI and EcoRV, shows that divalent metal ions are coordinated to the catalytic center of the enzyme (29–32). In order to examine further the requirement of divalent cation, namely  $\text{Mg}^{2+}$  in the PVBV NIa proteinase-catalyzed DNase reaction,  $\lambda$  phage DNA (5  $\mu\text{g}$ ) was incubated in the reaction buffer with purified NIa proteinase (1  $\mu\text{g}$ ) with increasing concentrations of EDTA (5–500  $\mu\text{M}$ ) for 30 min. DNA degradation was considerably retarded at EDTA concentrations above 50  $\mu\text{M}$  (Fig. 4A, lanes 3–7), and at 500  $\mu\text{M}$  all the DNA was present in the well as observed earlier. Next, the pH optimum of the DNase activity was determined by incubating  $\lambda$  DNA with NIa proteinase in the presence of 1 mM  $\text{MgCl}_2$  at 37  $^{\circ}\text{C}$  for 30 min at different pH values and analysis of the product by agarose gels. The DNase activity was maximal at pH >8.0 (Fig. 4B). To examine the effect of ionic strength on DNA binding and DNase activity,  $\lambda$  phage DNA was treated



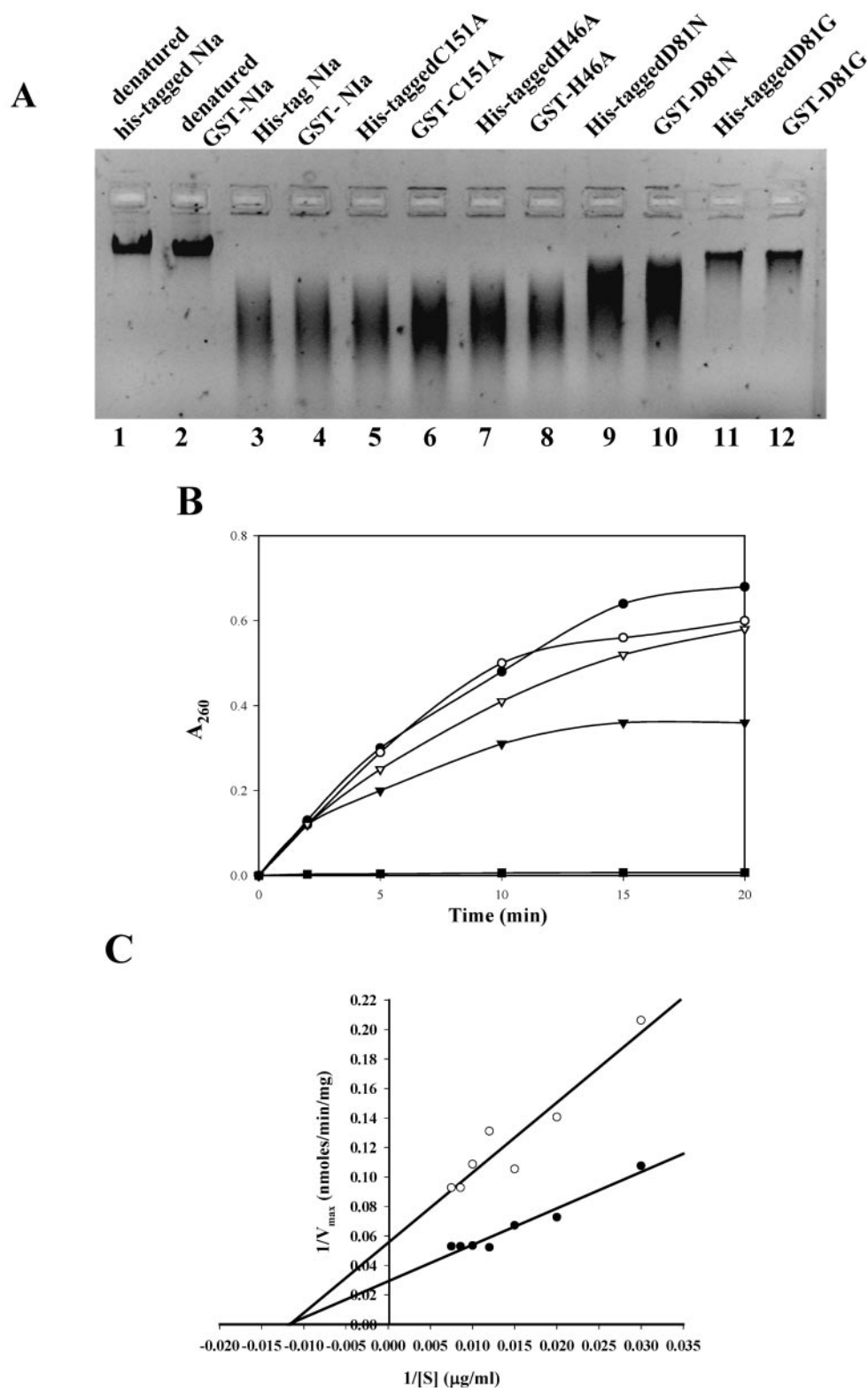
**FIG. 4. Effect of EDTA, pH, and NaCl on the DNase activity of the PVBV NIa proteinase.** *A*, effect of EDTA on the DNase activity of the PVBV NIa proteinase.  $\lambda$  phage DNA (5  $\mu$ g) and purified His-tagged PVBV NIa, 1  $\mu$ g, were incubated at 37  $^{\circ}$ C for 30 min in 20 mM Tris-HCl, pH 8.2, 1 mM MgCl<sub>2</sub> with increasing concentrations of EDTA (5–500  $\mu$ M). DNA cleavage/binding were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. *B*, effect of pH on DNase activity of PVBV NIa proteinase.  $\lambda$  DNA (lane 2) (5  $\mu$ g) and His-tagged PVBV NIa proteinase (1  $\mu$ g) were incubated at 37  $^{\circ}$ C in 20 mM sodium citrate buffer, pH 4.5 (lane 1), pH 5.0 (lane 2), 10 mM MES, pH 5.5 (lane 3), pH 6.0 (lane 4), 10 mM HEPES buffer, pH 6.5 (lane 5), pH 7.0 (lane 6), pH 7.5 (lane 7), 10 mM Tris-HCl, pH 8.0 (lane 8), pH 8.5 (lane 9), and 10 mM CAPS, pH 9.0 (lane 9). All the buffers included 1 mM MgCl<sub>2</sub>. The product formed was monitored by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining. *C*, effect of ionic strength on the DNA binding of the PVBV NIa proteinase.  $\lambda$  phage DNA (5  $\mu$ g) was incubated with 1  $\mu$ g of purified His-tagged PVBV NIa proteinase at 37  $^{\circ}$ C for 30 min in 20 mM Tris-HCl, pH 8.2, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA with decreasing concentrations of NaCl (150 to 50  $\mu$ M) and analyzed as before. Arrowheads indicate the mobility of the free DNA and DNA-protein complex. *D*, effect of NaCl on DNase activity.  $\lambda$  DNA (5  $\mu$ g) was incubated with purified PVBV NIa proteinase (1  $\mu$ g) for 15 min as described in the legend to Fig. 3D in presence of 1 mM MgCl<sub>2</sub> without EDTA. DNA binding/DNase activity was analyzed as before.

with His-tagged PVBV NIa proteinase at 37  $^{\circ}$ C for 0.5 h in the reaction buffer (20 mM Tris-HCl, pH 8.2, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA) at different concentrations of NaCl (50–150 mM). When the NaCl concentration was decreased, DNA-protein complex formation was favored, and intensity of the free DNA decreased (Fig. 4C, lanes 7 and 8). The  $I_{50}$  (50% of maximum binding) occurred at 80 mM NaCl. For TEV NIa,  $I_{50}$  of RNA binding (18) was found to be 112 mM. In order to examine how the DNA binding affects DNase activity, similar reactions were carried out with  $\lambda$  phage DNA in the absence of EDTA. As shown in Fig. 4E (lane 5), DNase activity was reduced to 50% of maximum at a NaCl concentration of 80 mM, clearly suggesting that DNA binding is essential for DNA degradation.

**Active Site Residues of NIa Proteinase Involved in DNase Function**—It was of interest to examine whether the active site residues involved in the DNA degradation were the same three catalytic triad residues involved in the proteolysis. We therefore studied the DNase activity of the His-tagged NIa proteinase and GST-fused NIa proteinase mutants by incubating  $\lambda$  phage DNA (5  $\mu$ g) with different mutant proteins (1  $\mu$ g) at 37  $^{\circ}$ C for 30 min, and the reaction products were analyzed by agarose gel electrophoresis as before. As shown in Fig. 5A, C151A (lanes 5 and 6) and H46A (lanes 7 and 8) were almost as active as the wild-type enzyme (lanes 3 and 4), whereas the activity of D81N was rather less (lanes 9 and 10). This suggested that His-46 and Cys-151 are not essential for DNase function, whereas Asp-81 could have some role. To further

establish the role of Asp-81, it was mutated to Gly such that any suboptimal interactions observed when Asp-81 was mutated to Asn could be eliminated. As shown in Fig. 4A (lanes 11 and 12), DNase activity was almost completely abolished in D81G mutant. It is also evident from these results that GST-fused NIa proteinase and mutants were functionally almost identical to that of His-tagged NIa proteinase and mutants (Fig. 5A). This also rules out any involvement of His tag or GST in the activity. Furthermore, the respective heat-denatured enzyme controls did not show any DNA degradation (Fig. 5A, lanes 1 and 2). Thus divalent metal ion and an active site aspartate seem to be essential for the DNase function of NIa proteinase.

To characterize further the DNase activity of His-tagged NIa proteinase, the kinetics of the enzymatic reaction was studied by measuring the liberated oligo- or mononucleotides. The un-cleaved substrate DNA was precipitated by treatment with acid-lanthanum reagent and was removed by centrifugation. The absorbance of the acid-soluble supernatant, containing oligo- and/or mononucleotides, was measured at 260 nm against a blank sample without enzyme. For a comparative analysis,  $\lambda$  phage DNA (50  $\mu$ g) was incubated separately with either of the wild-type or the mutant NIa proteins (5  $\mu$ g), and the increase in the absorbance was measured over a period of 20 min. As shown in Fig. 5B, the reaction was linear up to 15 min with the wild-type enzyme; H46A and C151A mutants were almost as active as the wild type NIa proteinase, whereas



**FIG. 5. Mutational analysis of DNase activity of NIa proteinase.** *A*, substrate  $\lambda$  phage DNA ( $5 \mu\text{g}$ ) and purified His-tagged NIa proteinase and its mutants ( $1 \mu\text{g}$ ) were incubated at  $37^\circ\text{C}$  in  $20 \text{ mM}$  Tris-HCl, pH 8.2, containing  $1 \text{ mM}$   $\text{MgCl}_2$ . The reaction was terminated after 30 min by addition of 6 times loading dye and was monitored by electrophoresis on  $0.7\%$  agarose gel followed by ethidium bromide staining. Lanes 3, 5, 7, 9, and 11 represent the reaction products of His-tagged NIa, NIa C151A, NIa H46A, NIa D81N, and NIa D81G proteinase, respectively; lanes 4, 6, 8, 10, and 12 represent the reaction products of GST-fused NIa, NIa C151A, NIa H46A, NIa D81N, and NIa D81G proteinase, respectively; samples with heat-denatured His-tagged NIa and GST-NIa added as negative control are represented in lanes 1 and 2, respectively. *B*,  $\lambda$  phage DNA ( $50 \mu\text{g}$ ) was incubated with purified His-tagged NIa proteinase ( $5 \mu\text{g}$ ) in  $20 \text{ mM}$  Tris-HCl, pH 8.2, containing  $1 \text{ mM}$   $\text{MgCl}_2$  at  $37^\circ\text{C}$  for different times, and the reaction was stopped by addition of equal volumes of acid lanthanum reagent ( $20 \text{ mM}$   $\text{La}(\text{NO}_3)_3$  in  $0.2 \text{ N}$  HCl). The precipitate was removed by centrifugation, and absorbance of the supernatant solution was measured at  $260 \text{ nm}$  against a blank sample without enzyme. WtNIa, D81N NIa, D81G NIa, H46A NIa, and C151A NIa are shown as  $\bullet$ ,  $\blacktriangledown$ ,  $\blacksquare$ ,  $\nabla$ , and  $\circ$ , respectively. Results are mean of five independent experiments. *C*, effect of substrate concentration. Substrate  $\lambda$  phage DNA ( $0$ – $150 \mu\text{g}$ ) was incubated with  $5 \mu\text{g}$  of purified His-tagged NIa proteinase as mentioned in *B*, and the nanomoles of product formed per min/mg enzyme (calculated as described under “Materials and Methods”) was plotted against the DNA concentration in the double reciprocal (Lineweaver-Burk) plot. WtNIa and D81N NIa, are shown as  $\bullet$  and  $\circ$ , respectively.



D81N was less active and the D81G mutant was inactive. Furthermore, the reaction was substrate concentration-dependent, and Fig. 5C shows the Lineweaver-Burk plot for the wild-type and D81N mutant NIa proteinase. The  $V_{\max}$  values of H46A and C151A (Table I) were almost same as wild-type PVBV NIa proteinase. However the  $V_{\max}$  was reduced by more than 2-fold for D81N mutant, and for the D81G mutant it was further reduced by almost 20-fold (Table I). The  $k_{\text{cat}}$  values of the wild-type and mutant enzymes are also shown in Table I. It is not possible to compare these values directly with the  $k_{\text{cat}}$  values for other DNases because the assay conditions are different. Bovine pancreatic DNase (5  $\mu\text{g}$ ) incubated with  $\lambda$  phage DNA (50  $\mu\text{g}$ ) under conditions identical to that used in the present study showed that it was 50 times more active than NIa proteinase (data not shown). The measured  $k_{\text{cat}}$  values appear to be low; this is because only the acid-soluble mono- and dinucleotides are measured in this assay and not the larger reaction products. However, it is clear from the agarose gel analysis of the samples (Fig. 3D) that almost complete degradation of the substrate DNA occurs in about 45 min. There was no apparent difference in the  $K_m$  values for the wild-type and the D81N mutant NIa proteinase (Fig. 5C). These results support our previous observation with agarose gel electrophoretic analysis of DNA degradation (Fig. 5A) that the DNase function of the NIa proteinase was intrinsic to the protein and not an artifact due to any contamination, as both the fusion proteins expressed and purified independently catalyze this reaction and the D81G mutant was inactive.

Recently, the x-ray crystal structures of the TEV NIa proteinase and its mutant C151A have been determined (12). We have examined the feasibility of replacing a double-stranded DNA molecule in place of the bound peptide in the C151A mutant enzyme (Protein Data Bank code 1lvb). It was possible to achieve superposition of alternate C- $\alpha$  atoms of the bound peptide and consecutive phosphorus atoms of an oligonucleotide in the B form for which coordinates are available in the Protein Data Bank (code 171d). However, short contacts were found at the C-terminal end and in a loop consisting of residues 170–174. It is conceivable that these short contacts are relieved by structural rearrangements upon DNA binding (data not shown).

**Detection of Nuclease Activity in the Infected Plant Nuclei**—As mentioned earlier, in *Potyriviruses* the NI proteins accumulate in the nucleus and form the nuclear inclusion bodies (2). It was of interest to examine whether the DNase function observed *in vitro* could have a physiological role. To check whether PVBV NIa protein indeed accumulates in the infected nuclei, Western blot analysis of the total nuclear proteins was carried out. As shown in Fig. 6A, polyclonal antiserum to PVBV NIa proteinase (9) could recognize a 49-kDa protein. This could correspond to NIa protein consisting of N-terminal viral genome-linked protein and the C-terminal proteinase domain. The NIa protein could be detected 7, 15, 21 days post-infection (Fig. 6A, lanes 2, 4, and 6). We then wanted to examine whether NIa protein present as nuclear inclusion in the infected plant was enzymatically active. Nuclear extract was prepared from the infected and healthy leaves and used for the assay. Proteinase activity of the infected nuclear extract was measured by incubating the His-tagged GFP substrate with the total nuclear extract from infected leaves and appearance of cleaved product was detected by Western blotting with anti-GFP antibodies. As shown in the Fig. 6B, nuclear extract from the healthy leaves could not cleave the substrate (Fig. 6B, lane 2) where as nuclear extract from infected leaves could cleave the substrate almost completely (Fig. 6B, lane 6). To check whether infected plant nuclear extract had DNase activity, we incu-

TABLE I  
Properties of the NIa proteinase and its mutants used in this study

Protein	$V_{\max}^a$	$k_{\text{cat}}^b$
PVBV NIa	38.4	0.0214
C151A NIa	38.9	0.0217
H46A NIa	37.0	0.0206
D 81N NIa	19.3	0.0107
D81G NIa	2.5	0.0014
TEV NIa	37.3	0.0208

<sup>a</sup> Values are expressed as nmol/min/mg.

<sup>b</sup> Values are expressed as  $\text{s}^{-1}$ .

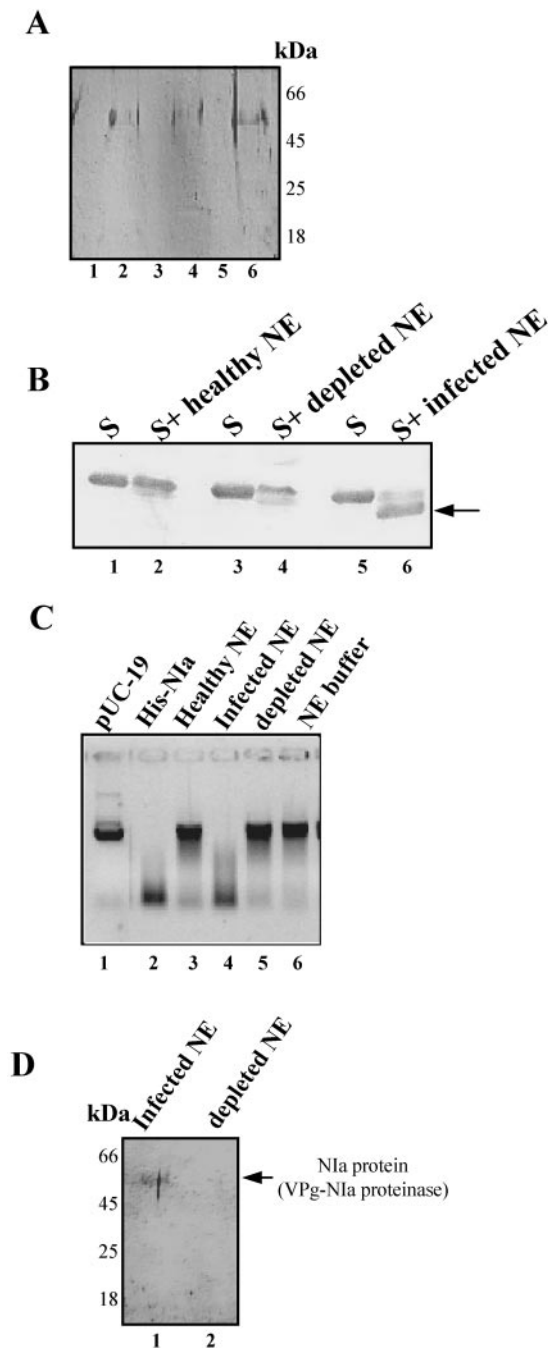
bated pUC-19 plasmid DNA with nuclear extract and monitored DNA degradation by agarose gel electrophoresis followed by ethidium bromide staining. As shown in Fig. 6C (lane 4), infected plant nuclear extract degraded the plasmid DNA, suggesting that infected nuclear extract had DNase activity. This activity is due to PVBV NIa protein and not probably due to any host protein because uninfected plant nuclear extract did not cause any DNA degradation (Fig. 6C, lane 3). To know whether the presence of NIa protein in the infected plant nuclei is responsible for the proteinase and DNase activities, NIa protein was immunodepleted from the infected nuclear extract by addition of rabbit polyclonal antiserum to NIa proteinase. Such immunodepleted nuclear extract did not show any band corresponding to the 49-kDa NIa protein suggesting that immunodepletion completely removed NI protein from the infected nuclear extract (Fig. 6D, lane 2). The immunodepleted infected nuclear extract was almost inactive with respect to the proteinase (Fig. 6B, lane 4) and DNase activity (Fig. 6C, lane 5). Therefore, these results clearly suggest that NI protein accumulated in the infected plant nucleus has both proteinase and DNase activities.

## DISCUSSION

The results presented in this paper demonstrate that PVBV NIa proteinase is a dual function enzyme as follows: 1) it has a proteinase activity with all the three catalytic triad residues Asp-46, Cys-151, and Asp-81 being essential for this function, and 2) a sequence nonspecific dsDNA degrading activity that requires the presence of divalent metal ions like  $\text{Mg}^{2+}$ . The binding of either ssDNA or dsDNA to the NIa proteinase does not require the presence of metal ions and is dependent on ionic strength. The binding is abolished at concentrations  $>100$  mM NaCl (Fig. 2), suggesting that the binding is probably through weak electrostatic interactions that are disrupted by high salt concentrations (Fig. 4). Similar results were also obtained with TEV NIa proteinase RNA binding (18). Prolonged incubation of 45 min in the presence of metal ion did not result in the cleavage of ssDNA by NIa proteinase, whereas within 15 min the dsDNA cleavage was obvious (Fig. 3, D and E). Thus NIa proteinase can act as metal ion-dependent nonspecific DNase. This activity is intrinsic to the NIa proteinase as it could also be demonstrated using the NIa proteinase from another potyvirus TEV and GST-fused PVBV NIa proteinase (Figs. 3–5).

Among the three catalytic triad residues, only Asp-81 seems to be essential for DNase function (Fig. 5). It is possible that Asp-81 is required for binding of the metal ion. Thus, when Asp-81 was mutated to Asn, only partial loss of activity was observed, but when it was mutated to Gly, the DNase activity was almost completely abolished (Fig. 5 and Table I).

Recently, the molecular mechanism of action of nonspecific DNase Vvn was proposed based on its crystal structure and biochemical characteristics (33). First a histidine (His-80 in Vvn) acts as a general base by activating a water molecule to attack the scissile phosphate. In the case of NIa proteinase, His-46 is not the residue involved and further mutational anal-



**FIG. 6. Characterization of NIa protein from infected plant nuclear extract.** *A*, Western blot analysis of PVBV NIa protein in nuclear extract from healthy (mock-infected) and infected plants at different time intervals post-inoculation. Lanes 2, 4, and 6 represent nuclear extract from 10, 15, and 20 days PVBV-infected leaves, respectively. Lanes 1, 3, and 5 represent Western analysis of nuclear extract of mock-infected plants. *B*, proteinase activity of infected plant nuclear extract. Chili plant nuclear extract (2 mg/ml) in 25 mM Tris-HCl, pH 8.2, containing 200 mM NaCl and 10% glycerol was incubated with GFP substrate at 37 °C for 4 h. Lanes 1, 3, and 5 represent GFP substrate. Cleavage was monitored by appearance of 30-kDa cleaved product after incubation with healthy plant nuclear extract (lane 2), immunodepleted nuclear extract from infected plant (lane 4), and infected plant nuclear extract (lane 6) in 12% SDS-PAGE and Western blotting using anti-GFP rabbit polyclonal antiserum. *C*, DNase activity of infected nuclear extract. Substrate pUC19 DNA (lane 1) and nuclear extract were incubated at 37 °C for 1 h in 20 mM Tris-HCl, pH 8.2, 1 mM MgCl<sub>2</sub> with recombinant His-tagged NIa proteinase (lane 2), healthy plant nuclear extract (lane 3), infected plant nuclear extract (lane 4), immunodepleted infected nuclear extract (lane 5), and nuclear extract buffer (lane 6), and the reaction was monitored by agarose gel electrophoresis as described in the legends to Fig. 3. *D*, immunodepletion of PVBV NIa from infected

ysis would be required to identify the general base. Next, a general acid provides a proton for the departing 3'-oxygen. It is suggested that the general acid could be a water molecule coordinated to Mg<sup>2+</sup> which is also involved in the stabilization of the phosphoanion transition state. Thus the cleavage is metal ion-mediated. The absolute requirement for Mg<sup>2+</sup> in the DNase function of NIa proteinase suggests that a similar mechanism may be operative even here. The Mg<sup>2+</sup> is coordinated to Glu-79 and Asn-127 in Vvn. Asp-81 in NIa proteinase could play a similar role as Glu-79. Other residues involved in metal coordination need to be identified. It was possible to accommodate a double-helical B-DNA segment in place of the peptide substrate in the crystal structure of TEV C151A mutant NIa proteinase, although it had some short contacts. These steric hindrances, however, could be relieved by appropriate conformational change. The B-DNA was bound to the active site via the minor groove, a characteristic feature of sequence nonspecific DNA binding. In general the overall mechanism of DNase function in NIa proteinase could be similar to that of nonspecific nuclease like Vvn.

Nuclease activity of proteinases has been reported earlier in the literature (34–37). It has been shown that along with multiendopeptidase activity, proteasomes are able to degrade RNA, albeit by an unknown mechanism. However, the DNase activity of any of the proteinases was not reported earlier. The active site of TEV NIa proteinase can accommodate dsDNA, and the experimental results presented in this paper establish that PVBV NIa proteinase can indeed function as a nonspecific DNase. Clearly, much work is needed to identify the other residues involved in the dsDNA degradation by PVBV NIa proteinase. There could be more than one acidic residue involved in the divalent metal coordination. Further investigation is also required to identify the residue, which acts as general base in the catalysis. As apparent from the results presented in the Fig. 6, NIa protein which accumulates in the nucleus of infected cells was capable of both proteinase and DNase function, thus emphasizing the physiological relevance and importance of the present findings.

The fact that potyviruses employ an apparently simple strategy of replication of RNA genome in cytoplasm makes them quite vulnerable to adaptive host defense responses. These viruses, on the other hand, have developed diverse strategies of host evasion that are detrimental to the host organism. The nuclease activity of potyvirus NIa proteinase demonstrated in this investigation might have important physiological implications in establishing effective virus infection. Accumulation of this protein at high concentration in the nucleus occurs in the late stage of the infection, when viral RNA synthesis is almost complete in the cytoplasm. Therefore, host genomic DNA degradation and thus killing the host could be useful for the virus in suppressing the host from mounting any systemic antiviral defense.

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nuclear extract. Total nuclear protein (2 mg/ml) was incubated for 2 h with 0.1 M Tris-HCl buffer, pH 7.5, containing 10 µg of affinity-purified rabbit polyclonal anti-NIa antibodies or nonspecific rabbit IgG (as a negative control). Subsequently, 100 µl of pre-washed protein A-Sepharose was added to the mix and incubated for 1 h at 4 °C. Immunodepleted supernatants were separated from the pellets by centrifugation at 12,000 × *g* for 30 s. When checked for the presence of PVBV NIa in these immunodepleted nuclear extracts, no 49-kDa band was detected (lane 2) as compared with nonspecific antiserum-depleted infected nuclear extract (lane 1) which showed the presence of a 49-kDa NIa protein band.

pRNIA C151A, and pRNIA D81N clones. We thank Gargi Meur Roy (Central University, Hyderabad) for the antibodies against GFP.

## REFERENCES

- Hull, R. (2002) *Matthews Plant Virology*, 4th Ed., A Harcourt Science and Technology Company, London, UK
- Riechmann, J. L., Lain, S., and Garcia, J. A. (1992) *J. Gen. Virol.* **73**, 1–16
- Anindya, R., Joseph, J., Gowri, T. D., and Savithri, H. S. (2004) *Arch. Virol.* **149**, 625–632
- Carrington, J. C., Cary, S. M., Parks, T. D., and Dougherty, W. G. (1989) *EMBO J.* **8**, 365–370
- Gorbalenya, A. E., Donchenko, A. P., Blinov, V. M., and Koonin, E. V. (1989) *FEBS Lett.* **243**, 103–114
- Kim, D. H., Hwang, D. C., Kang, B. H., Lew, J., and Choi, K. Y. (1996) *Virology* **221**, 245–249
- Kim, D. H., Hwang, D. C., Kang, B. H., Lew, J., Han, J., Song, B. D., and Choi, K. Y. (1996) *Virology* **226**, 183–190
- Garcia, J. A., Lain, S., Cervera, M. T., Riechmann, J. L., and Martin, M. T. (1990) *J. Gen. Virol.* **71**, 2773–2779
- Joseph, J., and Savithri, H. S. (2000) *Arch. Virol.* **145**, 2493–2502
- Carrington, J. C., Haldeman, R., Dolja, V. V., and Restrepo-Hartwig, M. A. (1993) *J. Virol.* **67**, 6995–7000
- Carrington, J. C., and Dougherty, W. G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3391–3395
- Phan, J., Zdanov, A., Evdokimov, A. G., Tropea, J. E., Peters, H. K., III, Kapust, R. B., Li, M., Wlodawer, A., and Waugh, D. S. (2002) *J. Biol. Chem.* **277**, 50564–50572
- Dougherty, W. G., Carrington, J. C., Cary, S. M., and Parks, T. D. (1988) *EMBO J.* **7**, 1281–1287
- Knuhtsen, H., Hiebert, E., and Purcifull, D. E. (1974) *Virology* **61**, 200–209
- Hajimorad, M. R., Ding, X. S., Flasiniski, S., Mahajan, S., Graff, E., Haldeman-Cahill, R., Carrington, J. C., and Cassidy, B. G. (1996) *Virology* **224**, 368–379
- Carrington, J. C., Freed, D. D., and Leinicke, A. J. (1991) *Plant Cell* **3**, 953–962
- Schaad, M. C., Haldeman-Cahill, R., Cronin, S., and Carrington, J. C. (1996) *J. Virol.* **70**, 7039–7048
- Daros, J. A., and Carrington, J. C. (1997) *Virology* **237**, 327–336
- Weiner, M. P., Costa, G. L., Schoettlin, W., Cline, J., Mathur, E., and Bauer, J. C. (1994) *Gene (Amst.)* **151**, 119–123
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
- Joseph, J., and Savithri, H. S. (1999) *Arch. Virol.* **144**, 1679–1687
- Patel, D., Frelinger, J., Goudsmit, J., and Kim, B. (2001) *BioTechniques* **31**, 1194, 1196, and 1198
- Rashid, N., Morikawa, M., Nagahisa, K., Kanaya, S., and Imanaka, T. (1997) *Nucleic Acids Res.* **25**, 719–726
- Warshaw, M. M., and Cantor, C. R. (1970) *Biopolymers* **9**, 1079–1103
- Cheah, K. S., and Osborne, D. J. (1977) *Biochem. J.* **163**, 141–144
- Parker, C. S., and Topol, J. (1984) *Cell* **37**, 273–283
- Hayes, J. D., and Pulford, D. J. (1995) *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600
- Oefner, C., and Suck, D. (1986) *J. Mol. Biol.* **192**, 605–632
- Beese, L. S., and Steitz, T. A. (1991) *EMBO J.* **10**, 25–33
- Volbeda, A., Lahm, A., Sakiyama, F., and Suck, D. (1991) *EMBO J.* **10**, 1607–1618
- Vipond, I. B., Baldwin, G. S., and Halford, S. E. (1995) *Biochemistry* **34**, 697–704
- Li, C. L., Hor, L. I., Chang, Z. F., Tsai, L. C., Yang, W. Z., and Yuan, H. S. (2003) *EMBO J.* **22**, 4014–4025
- Pouch, M. N., Petit, F., Buri, J., Briand, Y., and Schmid, H. P. (1995) *J. Biol. Chem.* **270**, 22023–22028
- Gautier-Bert, K., Murol, B., Jarrousse, A. S., Ballut, L., Badaoui, S., Petit, F., and Schmid, H. P. (2003) *Mol. Biol. Rep.* **30**, 1–7
- Jarrousse, A. S., Petit, F., Kreutzer-Schmid, C., Gaedigk, R., and Schmid, H. P. (1999) *J. Biol. Chem.* **274**, 5925–5930
- Petit, F., Jarrousse, A. S., Boissonnet, G., Dadet, M. H., Buri, J., Briand, Y., and Schmid, H. P. (1997) *Mol. Biol. Rep.* **24**, 113–117