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Molecular Cloning, Expression, and Purification of Nuclear Inclusion A Protease from Tobacco Vein Mottling Virus

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The gene encoding the C-terminal protease domain of the nuclear inclusion protein a (NIa) of tobacco vein mottling virus (TVMV) was cloned from an isolated virus particle and expressed as a fusion protein with glutathione S-transferase in *Escherichia coli* XL1-blue. The 27-kDa protease was purified from the fusion protein by glutathione affinity chromatography and Mono S chromatography. The purified protease exhibited the specific proteolytic activity towards the nonapeptide substrates, Ac-Glu-Asn-Asn-Val-Arg-Phe-Gln-Ser-Leu-amide and Ac-Arg-Glu-Thr-Val-Arg-Phe-Gln-Ser-Asp-amide, containing the junction sequences between P3 protein and cylindrical inclusion protein and between nuclear inclusion protein b and capsid protein, respectively. The K_m and k_{cat} values were about 0.2 mM and 0.071 s^{-1}, respectively, which were approximately five-fold lower than those obtained for the NIa protease of turnip mosaic potyvirus (TuMV), suggesting that the TVMV NIa protease is different in the binding affinity as well as in the catalytic power from the TuMV NIa protease. In contrast to the NIa proteases from TuMV and tobacco etch virus, the TVMV NIa protease was not autocatalytically cleaved into smaller proteins, indicating that the C-terminal truncation is not a common phenomenon occurring in all potyviral NIa proteases. These results suggest that the TVMV NIa protease has a unique biochemical property distinct from those of other potyviral proteases.

Keywords: Nuclear Inclusion Protein A; Potyvirus; TVMV; Viral Protease.

Introduction

Tobacco vein mottling virus (TVMV) is a member of the potyvirus group. The potyvirus belongs to the picornavirus superfamily of positive-strand RNA viruses (Riechmann *et al.*, 1992). The potyvirus contains an approximately 10-kb single-stranded positive-sense RNA as its genome (Allison *et al.*, 1986; Hellmann *et al.*, 1980; Riechmann *et al.*, 1992) (Fig. 1A). The potyviral genome encodes ca. 350-kDa polyprotein that is processed by three viral proteases to yield at least eight mature proteins (Riechmann *et al.*, 1992). Two proteases, protein 1 (P1) and the helper component protease (HC-Pro), located at the N-terminus of the polyprotein are released from the polyprotein by cleavage at their respective C-terminus autocatalytically (Carrington *et al.*, 1989; Mavankal and Rhoads, 1991; Verchot *et al.*, 1991) (Fig. 1A). The nuclear inclusion protein a (NIa) is responsible for the processing of the remaining regions of the polyprotein (Carrington and Dougherty, 1987; 1988; Hellmann *et al.*, 1988).

The NIa protein consists of two functional domains; the N-terminal genome-linked protein domain (VPg) and the C-terminal protease domain (Murphy and Rhoads, 1990; Parks and Dougherty, 1991). The NIa C-terminal 27-kDa protease, which plays a major role in polyprotein processing, cleaves polyprotein *in cis* at the junction between cylindrical inclusion protein (CI) and $6K_2$ protein, between the $6K_2$ protein and NIa, and between NIa and the nuclear inclusion protein b (NIb) or *in trans* at the junction between P3 and CI and between NIb and CP (Carrington and Dougherty, 1987; 1988; Carrington *et al.*, 1988; Riechamann *et al.*, 1992). The proteolytic activity of the

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Abbreviations: CI, cylindrical inclusion protein; HC-Pro, helper component protease; NIa, nuclear inclusion protein a; P1, protein 1; TEV, tobacco etch virus; TuMV, turnip mosaic virus; TVMV, tobacco vein mottling virus.

NIa protease for the respective cleavage site in the viral polyprotein might be responsible for the regulation of the levels of the viral gene products quantitatively or temporally. In this aspect, the activities of the NIa protease on their cleavage sites have been studied in such potyviruses as tobacco etch virus (TEV), plum pox virus (PPV) and turnip mosaic virus (TuMV) (Carrington and Dougherty, 1987; 1988; Carrington *et al.*, 1988; Dougherty and Parks, 1989; Garcia *et al.*, 1989; 1990; Kim and Choi, 1998; Rorrer *et al.*, 1992). The comparison of the biochemical properties of the potyviral NIa proteases will be valuable for the better understanding of the mechanism of the substrate recognition as well as the mechanism of the adaptation to the environment conditions of the host plants.

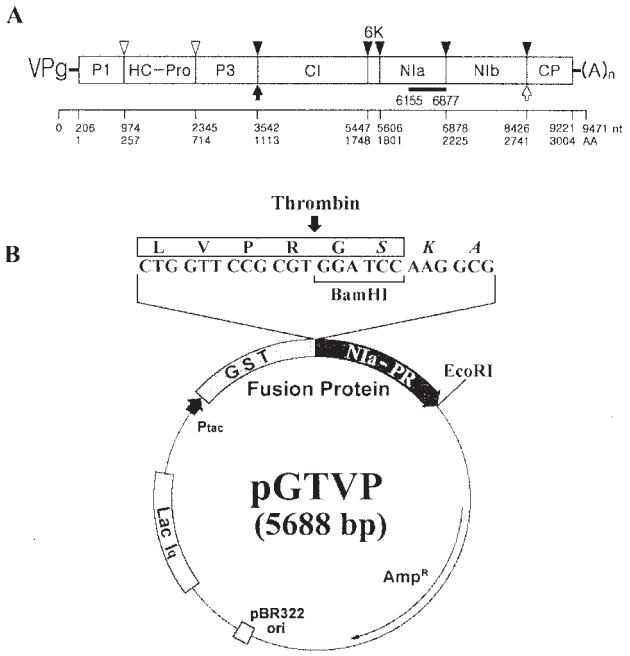

Fig. 1. A. Genomic structure of TVMV. The linear array of open boxes depicts the individual proteins that can be generated from proteolytic processing of the TVMV polyprotein. Untranslated regions are indicated as horizontal lines at each end of the map. Two open arrowheads indicate the cleavage sites processed by P1 and HC-Pro autocatalytically. The filled arrowheads indicate the cleavage sites processed by the NIa protease. The NIa protease gene is indicated as a black bar under the right region of NIa. Two peptide substrates used in this study are indicated by black and white arrows, respectively. **B.** Recombinant plasmids for the expression of the TVMV NIa protease gene. The italicized amino acids, S, K and A, represent the first three amino acids in the N-terminus of the NIa protease. The G is one additional amino acid attached to the N-terminus of the expressed NIa protease after the thrombin treatment. The thrombin recognition site is in a box and its cleavage site is indicated by an arrow.

In order to understand the biochemical property of the NIa protease from TVMV, the gene encoding the NIa 27-kDa C-terminal protease was cloned and expressed as a fusion form with glutathione S-transferase in *Escherichia coli*. The proteolytic activity was determined by both HPLC analysis and fluorescence spectrometry coupled with fluorescamine conjugation reaction. The protease was purified to homogeneity by affinity and ion-exchange chromatographies and its kinetic parameters have been obtained for two synthetic peptide substrates. The TVMV protease was distinct from other potyviral proteases in biochemical properties, suggesting that the potyvirus might have evolved to adapt to different environmental conditions of hosts.

Materials and Methods

Preparation of the viral RNA The TVMV RNA was purified from TVMV-propagated *Nicotina tabacum* according to the procedure as follows; 0.02 g of the TVMV-infected tobacco leaf tissue was homogenized and suspended with 0.5 M sodium phosphate buffer, pH 7.2, containing 1% β-mercaptoethanol. After the emulsion was centrifuged at 3,000 × *g* for 15 min, the supernatant was extracted with *n*-buthyl alcohol. The virus particle in the aqueous phase was extracted five times with water-saturated phenol/chloroform at pH 3.0. The viral RNA was precipitated with ethanol and then resuspended in the diethyl pyrocarbonate-containing distilled water.

Cloning of the gene encoding the NIa C-terminal 27-kDa protease cDNA was synthesized from the viral RNA with moloney murine leukemia virus reverse transcriptase (Life Technologies) using an oligonucleotide, VNIP-C: 5′-gGAAttc**tca**TTGAGTGCGGACCAAATCGTCCATGA-3′, specifying the sequence corresponding to the nucleotide sequence of TVMV genome from 6,852 to 6,877 (Domier *et al.*, 1986) as a primer, where the small letters represent the mismatched nucleotides and the bold letters represent the complementary sequence of the stop codon, TGA. The synthesized cDNA was used as a template for the polymerase chain reaction (PCR) with recombinant *Pfu* DNA polymerase (stratagene). VNIP-C and VNIP-N, 5′-AAGAAGTTGCATTCGgaTCcAAGGCGCTAC-3′, corresponding to the nucleotides from 6138 to 6167 in the TVMV genome, being used as primers for polymerase chain reaction, where the small letters represent the mismatched nucleotides. VNIP-C and VNIP-N contain the *Eco*RI and *Bam*HI restriction sites, respectively, for subsequent subcloning. The amplified DNA was digested partially by *Bam*HI and completely by *Eco*RI, and the resulting 729 bp DNA fragment was subcloned into pBluescript SK(+) phagemid (pSK(+)) (Stratagene). The sequence of the subcloned DNA was determined by the dideoxynucleotide chain termination method with an automated DNA sequencer (Applied Biosystem 373A, Perkin-Elmer Co.).

Construction of expression vector for the TVMV NIa 27-kDa protease In order to simplify the subcloning procedure, the *Bam*HI site in the cloned DNA encoding the NIa protease was eliminated by the site-directed mutagenesis (Kunkel *et al.*, 1987)

using a primer, 5′-CGTCAGGCTGTGtATCCCCAAA-ATATTTCC-3′, corresponding to the nucleotides from 6,635 to 6,664 in the TVMV genome. The mutated NIa protease gene was digested with *Bam*HI and *Eco*RI, and was subcloned into pSK(+) to construct pSTVP (+). The entire nucleotide sequences were determined to confirm the mutation without any change in other region of the NIa protease gene by the dideoxynucleotide chain termination method. The pSTVP (+) was digested with *Bam*HI and *Eco*RI, and the resulting inserted DNA fragment was subcloned into pGEX-KG (Guan and Dixon, 1991) to construct pGTVP (Fig. 1B).

Overexpression and purification of the recombinant TVMV NIa protease The NIa protease was over-expressed in *Escherichia coli* XL1-blue harboring pGTVP by inducing with 0.3 mM IPTG for 4 h at 27°C. The bacterial cells were harvested and resuspended with the lysis buffer (25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 10% glycerol, 2 mM EDTA and 1 mM DTT) containing the lysozyme (200 mg/ml. After incubation at 4°C for 30 min, the cell suspension was sonicated and the cell-debris was removed by centrifugation (12,000 × *g*, 4°C, 30 min). The supernatant was then bound to glutathione sepharose-4B resin (Pharmacia) for 30 min at 4°C. The resin was washed three times with the thrombin digestion buffer (50 mM Tris·HCl, pH 8.3, 100 mM NaCl and 2.5 mM $CaCl_2$). After the addition of two resin volume of the thrombin digestion buffer, the resin retaining the GST-NIa fusion protein was incubated with 20 μg of thrombin for 30 min at room temperature. The proteins released from the resin were collected and concentrated by Centriprep-10 (Amicon) with a cut-off of 10 kDa. The concentrated sample was diluted ten times with Mono S equilibration buffer (50 mM HEPES, pH 7.6, 10% glycerol, 1 mM EDTA and 1 mM DTT) and applied onto Mono S column (Pharmacia) equilibrated with the Mono S equilibration buffer, and then eluted with a linear gradient of 0–0.4 M NaCl. The fractions were collected and concentrated by using Centricon-10 (Amicon). The samples from each purification step were analyzed by 15% SDS-PAGE and stained with Coomassie brilliant blue G-250 staining.

Activity assay by HPLC Two synthetic nonapeptides, acetyl-Glu-Asn-Asn-Val-Arg-Phe-Gln-Ser-Leu-amide (VPSB) and acetyl-Arg-Glu-Thr-Val-Arg-Phe-Gln-Ser-Asp-amide (VPSF), were obtained from PeptidoGenic Inc. The VPSB and VPSF peptides contain the P3-CI and NIb-CP junction sequences, respectively (Fig. 1A) (Hellmann *et al.*, 1986; 1988). The typical reaction for the measurement of the proteolytic activity was carried out in the buffer containing 40 mM HEPES, pH 7.6, 150 mM KCl and 1 mM DTT with 0.2 mM peptide substrate and the NIa protease at 1 mM. The enzymatic reaction was carried out for 20 min at 25°C and stopped by adding 300 μl of 10% acetic acid before the product formation exceeded 5–15%. Two hundred microliters of the resulting mixture was analyzed by high performance liquid chromatography (Model HP1090, Hewlett-Packard) on a Vydac reverse-phase C_{18} column (4.6 mm ID × 25 cm) with an acetonitrile gradient and a flow rate of 1 ml/min. Absorbance at 220 nm was monitored to identify peaks from the products and the substrate.

Activity assay by fluorescamine reaction The proteolytic activity of TVMV NIa protease was also analyzed by use of fluorescamine which is reactive to the primary amine group (Udenfriend *et al.*, 1972; Weigele *et al.*, 1972). The reaction was carried out in the same way as described above for the HPLC analysis. The reaction was stopped by addition of 76 μl of 2% SDS and incubated with 100 μl of 0.01% fluorescamine for two hours before fluorescence measurement. The amount of the generated fluorescent amine-derivative was determined from the emission intensity at 468 nm with the excitation at 386 nm by a fluorescence spectrophotometer (Shimadzu RF-5000).

Results and Discussion

Nucleotide and amino acid sequence of the NIa protease gene The nucleotide sequences of the NIa C-terminal protease gene from TVMV were found to be identical to that previously reported one except one nucleotide at the position 57 from 5′-end of the NIa C-terminal protease gene with a change from G to C (Fig. 2) (Domier *et al.*, 1986). Such a change of the nucleotide resulted in the substitution of the tryptophan by cysteine at the amino acid position 19 from the N-terminus of the NIa protease. The three amino acids, His-46, Asp-81, and Cys-151, which were supposed to be the catalytic triad in the active site of the potyviral NIa proteases, were found to be conserved in the corresponding sites of the

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TCAAAGGCGCTACTTAAGGGAGTGCGAGATTTTAATCCAATCTCTGCTTGCGTATGCCTC    60
 S  K  A  L  L  K  G  V  R  D  F  N  P  I  S  A  C  V  C  L     20

CTTGAGAACTCCTCGGATGGGCATAGTGAGAGACTGTTTGGCATTGGTTTTGGCCCATAT   120
 L  E  N  S  S  D  G  H  S  E  R  L  F  G  I  G  F  G  P  Y     40

ATCATTGCCAACCAACATCTTTTTAGAAGGAACAATGGAGAGTTGACTATCAAAACCATG   180
 I  I  A  N  Q  H  L  F  R  R  N  N  G  E  L  T  I  K  T  M     60

CATGGTGAGTTCAAAGTCAAGAACTCAACACAATTGCAGATGAAACCAGTTGAGGGCAGA   240
 H  G  E  F  K  V  K  N  S  T  Q  L  Q  M  K  P  V  E  G  R     80

GACATAATAGTTATCAAAATGGCTAAGGACTTCCCACCATTCCCTCAAAAACTAAAATTC   300
 D  I  I  V  I  K  M  A  K  D  F  P  P  F  P  Q  K  L  K  F    100

AGACAGCCTACCATCAAAGATAGAGTGTGCATGGTATCCACAAATTTTCAGCAGAAAAGT   360
 R  Q  P  T  I  K  D  R  V  C  M  V  S  T  N  F  Q  Q  K  S    120

GTCTCTAGTCTAGTGTCTGAGTCATCACACATTGTGCATAAAGAGGACACTTCATTCTGG   420
 V  S  S  L  V  S  E  S  S  H  I  V  H  K  E  D  T  S  F  W    140

CAACACTGGATAACAACAAAGGATGGACAATGTGCAAGTCCGCTGGTTTCAATCATTGAT   480
 Q  H  W  I  T  T  K  D  G  Q  C  G  S  P  L  V  S  I  I  D    160

GGAAATATTTTGGGGATCCACAGCCTGACGCATACGACCAATGGTAGCAATTACTTCGTG   540
 G  N  I  L  G  I  H  S  L  T  H  T  T  N  G  S  N  Y  F  V    180

GAATTTCCTGAGAAGTTCGTAGCTACATATCTTGATGCCCCTGATGGTTGGTGCAAGAAT   600
 E  F  P  E  K  F  V  A  T  Y  L  D  A  A  D  G  W  C  K  N    200

TGGAAGTTCAATGCTGATAAGATCAGTTGGGGTTCCTTTACATTAGTTGAGGATGCGCCC   660
 W  K  F  N  A  D  K  I  S  W  G  S  F  T  L  V  E  D  A  P    220

GAAGATGACTTCATGGCCAAGAAAACTGTTGCCGCCATCATGGACGATTTGGTCCGCACT   720
 E  D  D  F  M  A  K  K  T  V  A  A  I  M  D  D  L  V  R  T    240

CAA                                                            723
 Q                                                              241
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Fig. 2. Nucleotide sequence of the cDNA encoding the TVMV NIa protease and its deduced amino acid sequence (GenBank accession number: AF222050). The nucleotide different from that reported previously (Domier *et al.*, 1986) and the resulting amino acid are shown in bold. The amino acids in boxes represent the putative catalytic triad of the active site in the NIa protease (Bazan and Flettrick, 1989). The underlined nucleotides indicate the internal *Bam*HI site.

TVMV NIa protease (Dougherty *et al*., 1990). The amino-acid sequence of the TVMV NIa protease was very similar to those of other potyviral NIa proteases from TEV, PVY, PPV, and TuMV with 70% in homology, which means that more than three amino acids out of five were identical in 70% of the amino acids (Allison *et al*., 1986; Domier *et al*., 1986; Lain *et al*., 1988; Nicolas and Laliberte, 1992; Robaglia *et al*., 1989).

Expression and purification of the NIa protease The recombinant plasmid, pGTVP, was designed to express the TVMV NIa protease as a fusion protein with a glutathione S-transferase (GST) at the N-terminus (Fig. 1B). The fusion protein, GST-NIa, was expressed at a high level in *E. coli* (Fig. 3). Most of the fusion proteins formed inclusion bodies at 37°C while they became soluble when the temperature was lowered down to 27°C. One major protein of 53 kDa, which corresponds to the expected size of the fusion protein of GST-NIa, was bound specifically to the glutathione-resin (Fig. 3, lane 3). This is in contrast with the result obtained for TuMV or TEV NIa protease (Kim *et al.*, 1995; Parks *et al*., 1995); the TuMV NIa protease was expressed as a doublet of 53- and 51-kDa proteins. The fusion protein was bound to glutathione affinity resin and digested with thrombin to release the 27-kDa NIa protease from the resin into supernatant while GST remained bound to the resin. The thrombin digestion of GST-NIa was expected to produce protease with one extra amino acid, glycine, at the N-terminus. The N-terminus of the purified NIa protease was confirmed by the N-terminal sequencing with Edman degradation method (data not shown). The NIa protease was purified to homogeneity by Mono S chromatography (Fig. 3, lane 5).

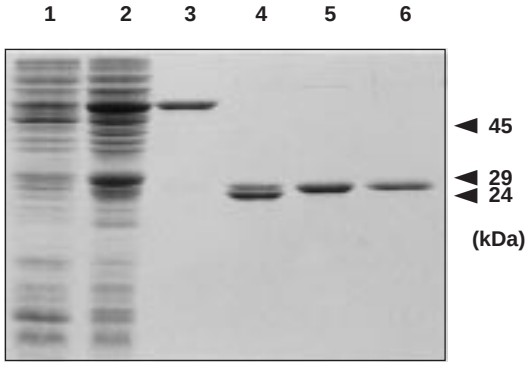

Fig. 3. SDS-PAGE analysis of the TVMV NIa 27-kDa protease during its purification. Each lane represents the uninduced (lane 1) and the induced (lane 2) cell lysates of *E. coli* XL1-blue harboring pGTVP, GST-NIa bound to glutathione resin (lane 3), the resin after thrombin-digestion of the proteins bound to the resin (lane 4), the protease released from the resin after thrombin-digestion of the proteins bound to the resin (lane 5), and the protease purified by Mono S chromatography (lane 6).

The concentration of the purified NIa protease was determined by measuring the absorbance at 280 nm (Pace *et al.*, 1995) or by Bradford assay. No significant difference was found between these two methods. Approximately 2 mg of the NIa protease could be obtained per liter of the bacterial culture.

The finding that TVMV NIa protease is not cleaved into smaller proteins implies that the TVMV NIa protease might be different from TuMV or TEV NIa protease in structural flexibility or stability. Otherwise, the self-cleavage occurring at the C-terminus of the TuMV and TEV NIa proteases might play a specific role for TuMV and TEV. The role of the autocatalytic truncation of the C-terminal residues in TuMV and TEV NIa proteases is not clear until now. The truncation of the twenty-four C-terminal amino acids of TEV NIa protease reduced the catalytic efficiency by approximately 20-fold, suggesting that the C-terminal residues might play a role in the substrate specificity (Parks *et al*., 1995). In contrast, the elimination of the C-terminal twenty amino acids of TuMV NIa protease did not significantly affect the *trans*-cleavage between $6K_1$ and CI or between NIb and CP as well as the *cis*-cleavage between NIa and NIb (Kim *et al*., 1996b; 1998), suggesting that the C-terminal twenty amino acids might not be important for the processing of the $6K_1$-CI, NIb-CP and NIa-NIb junction sequences. In order to understand the biological significance of the self-cleavage of the NIa protease more clearly, it is necessary to analyze the stability or flexibility of the NIa proteases as well as their effects on the proteolytic activity.

Proteolytic activities of the NIa protease The specific proteolytic activities of the TVMV NIa protease were determined by the use of either analytical HPLC or fluorescence spectrometry. For the HPLC method, the peptide substrate and product were separated by C_{18} reverse chromatography and monitored by measuring the absorbance at 220 nm (Fig. 4). The NIa protease was expected to cleave Gln-Ser peptide bond of VPSB to generate two products, acetyl-Glu-Asn-Asn-Val-Arg-Phe-Gln and Ser-Leu-amide. It could be shown that the product peak at the retention time of 7.6 min increased while the substrate peak at the retention time of 8.5 min decreased along with the incubation time. The cleavage of VPSF was analyzed in the same way as that used for VPSB. The areas of the two peaks were integrated to quantify the extent of the proteolytic reaction.

The catalytic activity of TVMV NIa protease was also analyzed using fluorescamine which is specifically reactive to a primary amine group. The peptide substrate with the acetylation at the N-terminus is not reactive to fluorescamine. If the peptide substrate is cleaved by the NIa protease, the primary amine group will be newly generated and reactive to fluorescamine. When the fluorescamine reacts with a primary amine group, it

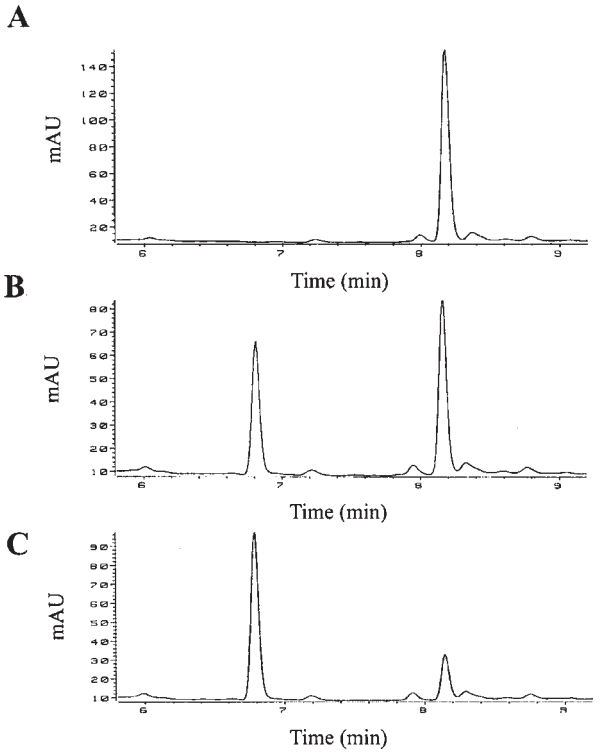

Fig. 4. HPLC analysis of the proteolytic activity of the purified NIa protease toward the nonapeptide substrate, Ac-Glu-Asn-Asn-Val-Arg-Phe-Gln-Ser-Leu-amide, containing the P3-CI junction sequence. The NIa protease was incubated with the peptide substrate for 0 (**A**), 60 (**B**), and 120 min (**C**). The product and the substrate were separated by use of the reverse phase C_{18} column and monitored by measuring the absorbance at 220 nm. The peaks at the retention times of 8.5 min and 7.6 min correspond to the substrate and the product, respectively.

generates a distinct fluorophore (Bohlen *et al.*, 1973) (Fig. 5A). The fluorescence of this product was measured at 466 nm after excitation at 386 nm. As shown in Fig. 5B, the emission level increased along with the incubation time of the reaction with the protease. The actual emission values of the product were obtained by the subtraction of the emission value obtained in the absence of the NIa protease. These values were converted to the concentrations of the product from the calibbration curve obtained by using glycine with a reactive group as a standard.

Kinetic parameters of the NIa protease The kinetic parameters, k_{cat} and K_m, were determined by the Lineweaver-Burk plot analysis of the proteolytic activities obtained from three independent measurements. The k_{cat} and K_m values were 0.216 mM and 0.071 s^{-1}, respectively, for VPSB while they were 0.217 mM and 0.079 s^{-1}, respectively, for VPSF (Table 1). Considering that the consensus cleavage-site sequence recognized by the TVMV NIa protease is Xaa-Xaa-Val-(Arg/Lys)-Phe-Gln*(Ser/Gly), where Xaa is any amino acid and the asterisk denotes the cleavage site (Domier *et al.*, 1986; Hellmann *et al.*, 1988), the similar values of the kinetic parameters for VPSF and VPSB might be due to the location of the same amino acids at the positions P4 through P′1 for both peptides. The kinetic parameters, K_m and k_{cat}, determined by the fluorescamine conjugation method were 0.171 mM and 0.103 s^{-1}, respectively, which were a little different from those obtained by the HPLC analysis. This might be due to the difference in emission intensities between glycine and the peptide in conjugation with fluorescamine. Actually, the emission levels of the

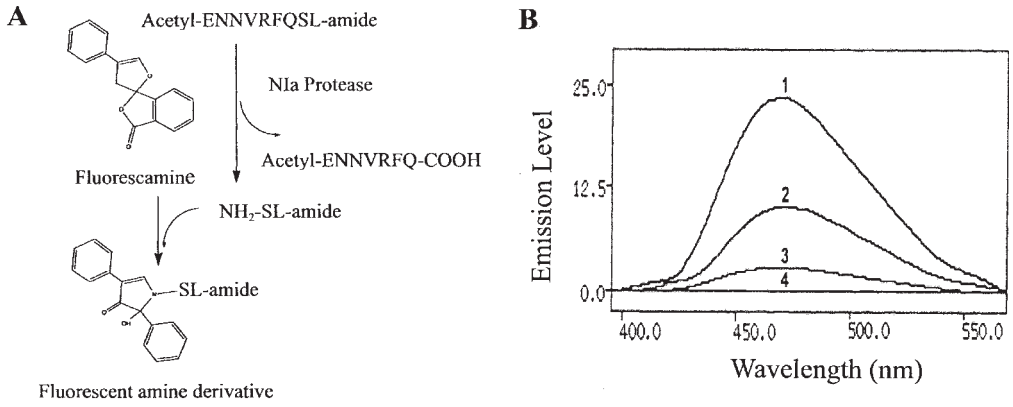

Fig. 5. Analysis of the proteolytic activity by fluorescamine conjugation reaction. **A.** The schematic diagram of the fluorescamine conjugation reaction. The reaction product of VPSF, NH_2-SL-amide, reacts with fluorescamine and is converted to a fluorescent amine derivative emitting maximally at 466 nm when excited at 386 nm. **B.** The emission spectra of the fluorescamine-conjugated product of NH_2-SL-amide, which was generated by the proteolytic activity of the NIa protease. The height of the emission spectrum is proportional to the amount of the generated product. The VPSF was incubated with the NIa protease for 60 (1), 30 (2), and 0 min (3), and the fluorescamine conjugation reaction was carried out for 2 h. The reaction mixture obtained in the absence of the NIa protease showed the residual emission level (4).

Table 1. Kinetic parameters of the TVMV NIa protease.

Assay Method	HPLC		Fluorospectrometry
Substrate[a]	VPSB (P3/CI)	VPSF (NIb/CP)	VPSB (P3/CI)
Range (mM)[b]	0.01–2.0	0.04–2.0	0.04–2.0
V_{max} (mM/min)	0.0042 ± 0.0001	0.0047 ± 0.0001	0.0062 ± 0.0003
K_m (mM)	0.216 ± 0.002	0.217 ± 0.011	0.171 ± 0.032
k_{cat} (sec^{-1})[c]	0.071 ± 0.003	0.079 ± 0.002	0.103 ± 0.005
k_{cat}/K_m ($M^{-1} \cdot sec^{-1}$)	327.1 ± 15.1	364.5 ± 18.5	603.9 ± 29.2

[a] The sequences of peptide substrates are described in Materials and Methods and their corresponding sites in the viral genome are indicated in parentheses.
[b] The numbers of substrate concentrations for VPSB (HPLC), VPSF (HPLC), and VPSB (fluorospectrometry) are fourteen, ten, and seven, respectively. The kinetic parameters by HPLC-VPSB were not different regardless of the ranges of the substrate, 0.04–2.0 mM and 0.01–2.0 mM.
[c] k_{cat} was calculated from V_{max} using the molecular weight of the NIa protease as 27-kDa.

Table 2. Comparison of the kinetic parameters of the potyviral NIa proteases and related chymotrypsin-like cysteine proteases.

Protease	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1} \cdot sec^{-1}$)	Reference
TVMV NIa 27-kDa[a]	0.216 ± 0.02	0.071 ± 0.003	327	This study
TEV NIa 49-kDa	0.069 ± 0.024	0.18 ± 0.022	2600	Parks *et al.*, 1995
TuMV NIa 27-kDa[b]	1.15 ± 0.16	0.33 ± 0.041	290[c]	Kim *et al.*, 1995
	2.22 ± 0.16	0.32 ± 0.01	144	Kim *et al.*, 1996a
Hepatitis A virus 3C	2.1 ± 0.5	1.8 ± 0.1	857	Malcolm *et al.*, 1992
Poliovirus 3C	1.3 ± 0.5	0.9[d]	692	Weidner and Dunn, 1991

[a] Kinetic constants of the TVMV NIa are the values from the first column of Table 1.
[b] Kinetic constants of the first row and the second row of the TuMV NIa protease were determined by using undecapeptide and nonapeptide, respectively.
[c] k_{cat}/K_m value was calculated from k_{cat} and K_m reported in the references cited.
[d] k_{cat} value was calculated from V_{max} data reported in the reference using the molecular weight of the picornaviral 3C protease as 20-kDa.

fluorophores generated from different amino acids are known to be quite different among them (Udenfriend *et al.*, 1972). Thus, the analysis of the proteolytic activity by use of the fluorescamine conjugation reaction should be modified to obtain the accurate kinetic parameters.

The overall catalytic efficiency, k_{cat}/K_m, of the TVMV NIa protease was similar to those of other potyviral NIa proteases and picornaviral 3C protease (Table 2). However, the K_m value of TVMV NIa protease was five or ten times lower than that of TuMV NIa protease while it was three times higher than that of TEV NIa protease. This difference might be attributed partly to the length of the peptide substrate used; the K_m value of the TEV NIa protease was obtained for the peptide of sixteen amino acids while K_m of the TuMV NIa protease was obtained for the undecapeptide. The longer peptide seems to have higher affinity for the NIa protease as judged by the K_m values of the TuMV NIa protease for different length peptides (unpublished data). It is noticeable that the TVMV NIa protease exhibits the lowest k_{cat} among the proteases in comparison.

On the basis of the primary and secondary structure analysis combined with the site-directed mutagenesis of the putative catalytic residues, it has been suggested that the potyviral NIa protease may take a chymotrypsin-like tertiary fold like that of the homologous picornaviral 3C protease (Bazan and Fletterick, 1988; 1989; Dougherty *et al*, 1989; Gorbalenya *et al.*, 1989; Kim *et al*., 1996c). The comparison of the kinetic parameters suggests that the catalytic powers of the potyviral NIa proteases are different among them and lower than those of hepatitis A virus and poliovirus 3C proteases. Such a difference should be understood in the point that the potyviral NIa protease might have evolved to optimize the viral replication rate in a given environment. The unusual biochemical property of the TuMV NIa protease with the low-temperature optimum activity has been considered to be acquired during evolution to meet with the environmental conditions of the host plants (Kim *et al*., 1996a).

In conclusion, the TVMV protease exhibits the biochemical properties without the C-terminal self-cleavage and with low turnover numbers which are distinct from those of other potyviral NIa proteases. Such differences might result from the adaptation of the virus to

the different environmental conditions exposed to the host plants. More detailed structural and biochemical analyses of the potyviral NIa proteases are necessary for a better understanding of their distinct biochemical properties and the mechanism of the adaptation to environmental conditions.

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