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Characterization of Active-Site Residues of the NIa Protease from Tobacco Vein Mottling Virus

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Nuclear inclusion a (NIa) protease of tobacco vein mottling virus is responsible for the processing of the viral polyprotein into functional proteins. In order to identify the active-site residues of the TVMV NIa protease, the putative active-site residues, His-46, Asp-81 and Cys-151, were mutated individually to generate H46R, H46A, D81E, D81N, C151S, and C151A, and their mutational effects on the proteolytic activities were examined. Proteolytic activity was completely abolished by the mutations of H46R, H46A, D81N, and C151A, suggesting that the three residues are crucial for catalysis. The mutation of D81E decreased k_{cat} marginally by about 4.7-fold and increased K_m by about 8-fold, suggesting that the aspartic acid at position 81 is important for substrate binding but can be substituted by glutamate without any significant decrease in catalysis. The replacement of Cys-151 by Ser to mimic the catalytic triad of chymotrypsin-like serine protease resulted in the drastic decrease in k_{cat} by about 1,260-fold. This result might be due to the difference of the active-site geometry between the NIa protease and chymotrypsin. The protease exhibited a bell-shaped pH-dependent profile with a maximum activity approximately at pH 8.3 and with the abrupt changes at the respective pK_a values of approximately 6.6 and 9.2, implying the involvement of a histidine residue in catalysis. Taken together, these results demonstrate that the three residues, His-46, Asp-81, and Cys-151, play a crucial role in catalysis of the TVMV NIa protease.

Keywords: NIa Protease; Tobacco Vein Mottling Virus.

Introduction

Tobacco vein mottling virus (TVMV) is a member of the Potyviridae that belongs to the picornavirus superfamily of positive-strand RNA viruses (Domier *et al*., 1986; Pirone and Gooding, 1973). The genomic RNA is translated into one polyprotein in a host cell, which is then processed into functional proteins by three viral proteases: protein 1 (P1), helper component protease (HC-Pro), and nuclear inclusion protein a (NIa). The P1 and HC-Pro proteases cleave only their respective C-termini (Carrington *et al*., 1989; Verchot *et al*., 1991). The NIa protease is responsible for processing the C-terminal two-thirds of the viral polyprotein (Carrington and Dougherty, 1987). The NIa protease consists of two functional domains: the 22-kDa VPg (viral protein genome-linked) domain at the N-terminus and the 27-kDa protease domain at the C-terminus (Dougherty and Parks, 1991; Murphy and Rhoads, 1990). On the basis of its sequence similarity, the NIa protease has been related to the chymotrypsin-like serine protease (Bazan and Fletterick, 1988; Gorbalenya *et al*., 1989).

In spite of the importance of the NIa protease in viral replication, the biochemical properties of the protease have not been extensively studied until now (Kim and Choi, 1998). Bazan and Fletterick (1988; 1989) and Gorbalenya *et al*. (1989) have independently suggested in their molecular modeling studies that the potyvirus NIa proteases possess structural motifs similar to that of the chymotrypsin-like serine proteases. The sequence

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Abbreviations: DTT, dithiothreitol; IPTG, isopropyl α-thiogalactopyranoside; NIa, nuclear inclusion protein a; TEV, tobacco etch virus; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; TuMV, turnip mosaic virus; TVMV, tobacco vein mottling virus.

alignment of the potyviral NIa proteases with the serine proteases could help predict the active-site residues involved in the catalysis since the serine proteases possess a catalytic triad consisting of histidine, aspartic acid, and serine. The primary sequence, the locations of the catalytic triad residues, and the role in the viral replication of the NIa protease were found to be very similar to those of the picornavirus 3C proteases, which are known to be similar to the chymotrypsin-like serine proteases (Allaire *et al*., 1994; Bazan and Fletterick, 1988). Recently, structural analysis of the picornaviral 3C protease from hepatitis A virus and human rhinovirus-14 by X-ray crystallography showed that their structures are very similar to chymotrypsin (Allaire *et al*., 1994; Matthews *et al*., 1994). The NIa protease from tobacco etch virus (TEV) which is a member of the potyvirus group has been found to be inhibited by TPCK and cysteine protease inhibitors, suggesting that the protease is a cysteine protease similar to the serine proteases in its active-site geometry (Dougherty *et al*., 1989). The inhibition and the site-directed mutagenesis studies could identify the catalytic triad of the NIa protease of tobacco etch virus (TEV), His-46, Asp-81, and Cys-151 (Bazan and Fletterick, 1989; Dougherty *et al*., 1989). The corresponding residues of the NIa protease from turnip mosaic virus (TuMV) and plum pox virus (PPV) were also found to be crucial for the catalysis (Garcia *et al*., 1989; 1990; Kim *et al*., 1995; 1996b).

In order to identify the active-site residues of the TVMV NIa protease, the putative active-site residues, His-46, Asp-81, and Cys-151, were mutated individually. The mutational effects were then analyzed by determining the kinetic parameters for catalysis. The mutations abolished or drastically decreased the catalytic activity, suggesting that the residues are crucial for catalysis. The pH-dependence profile of the catalytic activity of the protease exhibited a bell-shaped profile with an implication of the involvement of a histidine residue in catalysis. Our studies demonstrated that the three active-site residues play an essential role in catalysis like the catalytic triad of the chymotrypsine-like serine proteases.

Materials and Methods

Site-directed mutagenesis His46 was changed to Ala and Arg to make H46A and H46R, respectively; Asp81 was changed to Asn and Glu to make D81N and D81E, respectively; and Cys151 was changed to Ala and Ser to make C151A and C151S, respectively, according to the procedure described previously (Kunkel, 1985). Single-stranded uracil-containing template DNA complementary to the coding strand of the protease gene was obtained from pSTVP (+) (Hwang *et al*., 2000), a pBluescript SK(−) plasmid containing the TVMV NIa protease gene, which had been introduced in *Escherichia coli* RZ1032 after infection with the helper phage M13K07 (Amersham Pharmacia Biotech). The six oligonucleotides listed in Table 1 were synthesized and used as primers for the respective mutagenesis. The oligonucleotides were designed to generate the restriction site that can be utilized to identify the mutation in an easy way. The mutated gene sequence was confirmed by the dideoxynucleotide chain termination method with an automated DNA sequencer (Applied Biosystem 373A, Perkin-Elmer Co.). The mutated gene in pSK(−) was digested with *Eco*RI and *Bam*HI to isolate the inserted DNA fragment and then subcloned into the same sites of pGEX-KG (Guan and Dixon, 1991).

Expression and purification of the NIa proteases The expression and purification of the mutant proteases were performed according to the procedure described previously (Hwang *et al*., 2000); briefly, the cloned gene encoding the TVMV NIa protease in pGEX-KG (Guan and Dixon, 1991) was produced as a fusion protein with glutathione S-transferase (GST) in *E. coli* XL1-blue by induction with the addition of 0.3 mM of IPTG for 4 h at 27°C. The expressed GST-fusion

Table 1. Oligonucleotides for site-directed mutagenesis of TVMV NIa protease.

Mutant[a]	Location of primer on TVMV RNA[b]	Sequence of mutagenic oligonucleotide, 5′ → 3′[c]	Restriction endonuclease
H46A	6275–6311	C ATT GTT CCT TCT AAA tAa **agc** TTG GTT GGC AAT GAT	*Hin*dIII
H46R	6274–6311	C ATT GTT CCT TCT AAA tAa **gcG** cTG GTT GGC AAT GAT A	*Eco*47III
D81N	6383–6415	CAT TTT GAT AAC TAT aAT **att** TCT GCC CTC AAC	*Ssp*I
D81E	6379–6413	T TTT GAT AAC TAT TAT t**TC** gCg aCC CTC AAC TGG T	*Spo*I
C151A	6588–6618	AGC CGG ACT TCC **Agc** TTG gCC ATC CTT TGT TG	*Bal*I
C151S	6592–6619	C AGC CGG ACT TCC **ggA** TTG TCC ATC CTT T	*Acc*III

The boxes indicate the sites recognized by the corresponding restriction enzymes. The small letters indicate the mismatched nucleotides. The bold letters indicate the codon which corresponds to the mutated amino acids. The oligonucleotides are complementary to the coding sequence of the protease gene.

[a] Amino acids are indicated using the standard one letter code.

[b] Nucleotide numbers are based on the TVMV RNA sequence (Domier *et al*., 1986).

[c] Each oligonucleotide was designed to generate one restriction site.

Table 2. Kinetic parameters of the wild-type and its mutant enzymes[a].

Enzyme type	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Relative value (%)[b]		
				K_m	k_{cat}	k_{cat}/K_m
Wild-type	0.22 ± 0.01	0.071 ± 0.003	327.13 ± 15.05	100	100	100
H46R	N.D.[c]					
H46A	N.D.					
D81E	1.75 ± 0.15	0.015 ± 0.001	8.68 ± 0.74	795	21.43	2.65
D81N	N.D.					
C151S	0.11 ± 0.01	$(5.65 \pm 0.46) \times 10^{-5}$	0.52 ± 0.04	2.42	0.08	0.16
C151A	N.D.					

[a] The kinetic parameters were obtained for the wild type at 1 μM and for D81E and C151S at 10 μM.
[b] The relative values represent the percentage of the kinetic constants relative to that of the wild type protease.
[c] The proteolytic activity was not detected.

NIa protein was specifically bound to Glutathione Sepharose-4B resin (Amersham Pharmacia Biotech) separately from other *E. coli* proteins. The NIa protease was released from the affinity resin by the digestion with thrombin that cleaves the junction between the GST and the NIa protease. After concentration, the fraction of the NIa protease was applied onto a Mono-S 5/5 HR column (Amersham Pharmacia Biotech), and eluted with a linear gradient of 0–0.4 M NaCl as the final step. About 2 mg of the mutant NIa protease was purified from 1 l of bacterial culture.

Determination of kinetic parameters The proteolytic activity of the mutant enzymes was determined using a nonapeptide substrate, acetyl-Glu-Asn-Asn-Val-Arg-Phe-Gln-Ser-Leu-amide, containing the junction sequence between protein 3 (P3) and the cylindrical inclusion protein (CI) by HPLC analysis as described previously (Hwang *et al.*, 2000). Since the catalytic activities of the mutant enzymes were significantly lower than that of the wild-type enzyme, the higher concentrations of the proteases as well as the extended reaction times were used to adjust product formation to not exceeding 5–15% of the used substrate. The K_m and k_{cat} values of the mutant NIa proteases were determined by analyzing the Lineawer-Burk plots and the Michealis-Menten equation in a range of substrate concentration from 0.2 to 2 mM. Activities were determined by averaging the results from more than three independent experiments for the determination of the kinetic constants.

pH dependence of the catalytic activity The effect of pH on the proteolytic activity of the TVMV NIa protease was examined by measuring the catalytic activity at different pH conditions using 40 mM of MES (pH 5.48–6.48), HEPES (pH 7.0–7.48), Tricine (pH 7.0–9.0), Tris (pH 7.1–9.52), Sodium carbonate/bicarbonate (pH 9.0–10.0) or CAPS (pH 10.5–11.0) with 150 mM KCl and 1 mM of DTT in the buffer solution. The reaction was carried out with the above nonapeptide substrate at a 0.2 mM concentration. The reaction was started by the addition of NIa protease to the buffer containing the peptide substrate, incubated for 20 min at 25°C, and then stopped by the addition of 300 μl of 10% acetic acid.

Results and Discussion

Mutational effects on k_{cat} and k_m The mutations of H46A, H46R, D81N and C151A abolished the catalytic activity almost completely, suggesting that the three residues, His-46, Asp-81, and Cys-151, are crucial for catalysis. On the other hand, k_{cat} was decreased marginally by about 4.7-fold for D81E, suggesting that Asp81 can be replaced by a glutamate without a significant decrease in activity. The abolishen of the activity by the D81N mutation suggests that the carboxyl group at position 81 is important for catalysis. But, the K_m was increased by about 8-fold for D81E, suggesting that residue 81 is important for substrate binding. In trypsin, the active-site aspartic acid plays a role in polarizing the imidazole ring of the active-site histine residue. Even though it is not clear whether Asp81 is responsible for polarizing the imidazole ring of His46, the more significant effect of the D81E mutation on K_m than on k_{cat} implies that Asp81 might play an important role in substrate recognition (Carter and Wells, 1988; Craik *et al.*, 1987).

The mutation of C151S drastically decreased k_{cat} by about 1,260-fold. This result suggests that the replacement of the active-site cysteine by serine cannot generate the active-site environment of chymotrypsin. The TEV and TuMV NIa proteases have been found to be inhibited by TPCK more efficiently than by TLCK, suggesting that the geometry of the active site of the NIa protease resembles that of chymotrypsin (Dougherty *et al.*, 1989; Kim *et al.*, 1996c). This indicates that the thiol group of Cys151 in the NIa protease could be replaced by the hydroxyl group of serine. However, in the TEV NIa protease, the mutation of cysteine to serine resulted in marginal catalytic activity (Dougherty *et al.*, 1989). Similarly, the substitution of the active site serine residue of trypsin to cysteine eliminated the activity by a factor of approximately 10^5 (Higaki *et al.*,

Fig. 1. Alignments of the amino acid sequences of the potyviral NIa proteases. The residues conserved as homologous in at least 6 species were shaded. Residues belonging to one of the following groups were scored as homologous: A, V, L, I; D, E; N, Q; S, T; K, R; F, Y, W; M, C. The bold letters indicate the putative active-site residues. Dashes indicate gaps inserted to maximize alignment. The numbers beneath the sequences indicate the residue locations of the TVMV NIa protease. Abbreviations (references): TVMV, tobacco vein mottling virus (Domier *et al*., 1986); TEV, tobacco etch virus (Allison *et al*., 1986); TuMV, turnip mosaic virus (Nicolas and Laliberte, 1992); SMV, soybean mosaic virus (Jayaram *et al*., 1992); JGMV, johnson grass mosaic virus (Gough and Shukla, 1992); PVY, potato virus Y (Robaglia *et al*., 1989); PPV, plum pox virus (Lain *et al*., 1989); PRSV, papaya ringspot virus (Yeh *et al*., 1992); PSbMV, pea seed-borne mosaic virus (Johansen *et al*., 1991), and PepMoV, pepper mottle virus (Vance *et al*., 1992).

1987). This might be due to the difference in the active site geometry surrounding the nucleophilic residue between the cysteine- and serine-proteases. The modification of the nucleophilic cysteine to serine may be accompanied by other changes that contribute to a new active site environment. This result implies that the catalytic mechanism might be different from those of the typical serine proteases (Dougherty and Semler, 1993).

pH dependence of the catalytic activity of TVMV NIa protease The effect of pH on the proteolytic activity of the TVMV NIa protease was found to exhibit a bell-shaped profile with a maximum at approximately pH 8.1 (Fig. 2). The activity increased from pH 5.5 to 8.0 and decreased from pH 8.2 to 11.0 with pK_a values of approximately 6.6 and 9.2. The pK_a value of 6.6 is similar to that of chymotrypsin (pK_a = ~7) (Dugas, 1996). Most serine proteases including chymotrypsin exhibit a sigmoidal curve for the pH-dependence of catalytic activity. Hepatitis A viral 3C protease, which is also predicted to have a chymotrypsin-like motif (Malcolm, 1995), shows a sigmoidal curve of pH-

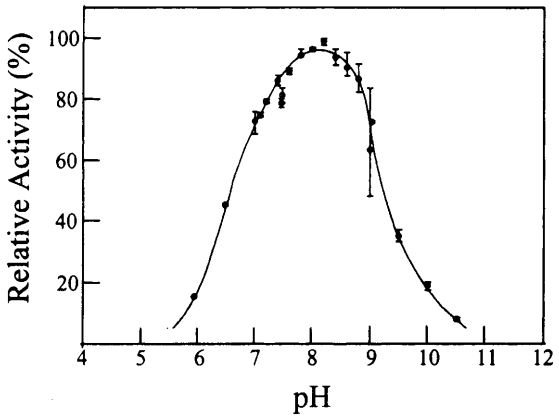

Fig. 2. pH dependence of the catalytic activity of the TVMV NIa protease. Proteolytic activity was measured using the synthetic peptide, actyl-Glu-Asn-Asn-Val-Arg-Phe-Gln-Ser-Leu-amide, by the HPLC method. The buffer was selected according to its proper buffering range of pH; MES (pH 5.48 to 6.48), HEPES (pH 7.0 to 7.48), Tricine (pH 7.0 to 9.0), Tris (pH 7.1 to 9.52), sodium carbonate/bicarbonate (pH 9.0 to 10.0), and CAPS (pH 10.5 to 11.0). The relative activity represents the percentage of the catalytic activity relative to the highest one as 100. The error bars indicate the standard deviation from three independent measurements.

dependence with a pK_a of 6.2 ($\pm$0.2) (Jewell *et al.*, 1992). The pK_a value of the TVMV NIa protease is different from those of the cysteine proteases such as papain whose pH dependence activity profile is bell-shaped with pK_a values of 4.0 and 8.0 (Storer and Menard, 1994), suggesting that the residues involved in the catalysis of the potyviral NIa protease might be different from those of the cysteine proteases. Assuming that the TVMV NIa protease has a structural motif similar to that of chymotrypsin, a pK_a of 6.6 strongly implicates the presence of a histidine. The origin of the higher pK_a of 9.2 might reflect a conformational change induced by low protonation.

Recently, the pH dependence of the catalytic activity of TuMV NIa protease was reported to have a pK_a of 7.4, which is quite different from that of TVMV NIa protease (Menard *et al.*, 1995). This implies that the active-site environment or geometry of the TVMV NIa protease might be different from the TuMV NIa protease. In addition to the difference in pK_a, these two enzymes are also different in the C-terminal self-cleavage pattern and the turnover number (Hwang *et al.*, 2000). Previously, the C-terminal residues of the NIa protease were observed to be cleaved autocatalytically in TEV and TuMV while they were not cleaved in TVMV (Kim *et al.*, 1995; Parks *et al.*, 1995). Even though the role of the C-terminal self-cleavage is not clear in proteolytic function or viral replication (Kim *et al.*, 1996a; 1996b; 1998), no observation of C-terminal self-cleavage in the TVMV NIa protease implies that this C-terminal self-cleavage does not generally occur in all potyviral NIa proteases. It is not clear whether the C-terminal self-cleavage is related to the low turnover number or the low pK_a value of the TVMV NIa protease.

It should be taken into consideration, however, that the loss of proteolytic activity on the substitution of a residue dose not necessarily mean that the residue is part of the active site of the protease. The residue involved might alternatively be involved in substrate binding or in maintaining the overall structure of the enzyme. For His-46 of the TEV NIa protease, its catalytic role was clearly confirmed by the experiment showing that among the respective mutagenesis of three histidines the mutation of His-46 abolished the proteolytic activity while two other mutations, H28Y and H61Y, did not influence the activity (Dougherty *et al.*, 1989).

In conclusion, the mutational studies suggest that the three residues, His-46, Asp-81 and Cys-151, may comprise the catalytic triad of the TVMV NIa protease. The pH-dependent activity profile of TVMV NIa protease implicates the involvement of a histidine residue in catalysis. The significant decrease in k_{cat} by the mutation of C151S suggests that the active-site environment of TVMV NIa protease is quite different from that of the serine proteases. The same composition of the catalytic triad among the potyviral NIa proteases supports that they have an analogous catalytic mechanism. The detailed three-dimensional structure of the NIa protease will contribute to a better understanding of the active-site geometry different from that of the chymotrypsin-like serine proteases.

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