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Proteolytic activity of the plum pox potyvirus NI_a-protein on excess of natural and artificial substrates in *Escherichia coli*

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The plum pox potyvirus (PPV) NI_a protease expressed from a medium copy number plasmid was able to process an excess of substrate expressed from a high copy number plasmid, in a binary *Escherichia coli* expression system. The ΔB7 NI_a protease mutant only partially processed the NI_b-CP junction but its efficiency was independent of the amount of substrate. The ΔB7 mutant essentially did not recognize an artificial cleavage site which was quite efficiently recognized by the wild-type protease. No competitive inhibition of the proteolytic activity by the presence of excess of different protease mutants was observed.

Potyvirus; Viral protease; Cleavage site, artificial; Expression system; *(E. coli)*

1. INTRODUCTION

The participation of virus-encoded proteases in the processing of polyprotein precursors has been described in a large number of different animal and plant RNA containing viruses [1]. Because of this critical role, together with their strong specificity, the proteases have been considered possible targets for antiviral action [2]. The genomic RNA of plant potyviruses is translated in a polyprotein of about 350 kDa which is processed by at least two viral encoded proteases [3,4]. One of them, known as NI_a-type or 49 kDa protease, recognizes sites characterized by highly conserved heptapeptide sequences [5–7]. The heptapeptide sequence corresponding to the tobacco etch virus (TEV) 58–30 kDa [8] or the PPV NI_b-CP [9] cleavage sites inserted into nonspecific protein regions were efficiently recognized by their respective proteases.

As a first step in the study of different ways to inhibit the viral proteases we have analyzed in an *E. coli* expression system the activity of the PPV NI_a-type protein in the presence of an excess of natural and artificial substrates. The activity of the wild-type protease synthesized together with mutant proteins which have different levels of proteolytic activity has also been studied.

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Abbreviations: aa, amino acids; nt, nucleotides; ORF, open reading frame; CP, capsid protein; NI_a and NI_b, nuclear inclusion protein a and b; (m)Ab, (monoclonal) antibody

2. MATERIALS AND METHODS

2.1. *Bacterial strain and plasmids*

All cloning of plasmids was done in *E. coli* JM109 [10].

Plasmids pPPVS20 and pPPVS20ΔB7 (figs 1C and 2C) were obtained by insertion of the *Sal*I-*Pst*I fragment (containing the 3′ terminal region of PPV cDNA from nt 3627) of pPPV12 or pPPV12ΔB7 [7] in the corresponding sites of the polylinker of pSU8 (a derivative of pSU2716 [11], with unique *Eco*RI and *Acc*I sites; F. de la Cruz, personal communication). Plasmids pPPV30, pPPV30sP16, pPPV30Δcp and pPPV30mP5 (figs 1C and 2C) were obtained by ligation of the *Eco*RV-*Eco*RI fragment containing nt 8514–9319 of PPV cDNA sequence from pPPV12, pPPV12sP16, pPPV12Δcp and pPPV12mP5 [9], respectively, with pUC19 [11] digested with *Sma*I and *Eco*RI. Plasmids pPPV23ΔB2, ΔB3, ΔB5, ΔB7 and ΔB10 (fig.3C) were obtained by replacing the sequence of pPPV30 from the *Hin*cII site of the polylinker to the *Sac*I site at position 9020 of the PPV genome with the *Sma*I-*Sac*I fragment (PPV nt from 4237 to 9020) of pPPV12ΔB2, ΔB3, ΔB5, ΔB7 and ΔB10 [9], respectively. The numbers of the PPV sequence are according to [12].

In all the cases the PPV ORF was placed in phase with the first codons of the β-galactosidase gene of pSU8 or pUC19. This ORF ended at the termination codon of the PPV polyprotein in pPPVS20 and pPPVS20ΔB7. In the rest of the plasmids it lacked the 81 last codons of the CP cistron and was fused to pUC19 sequences (48 additional codons).

2.2. *Immunological detection of PPV polypeptides*

The preparation of the extracts, separation in SDS-polyacrylamide gels and immunodetection of the proteins transferred to nitrocellulose membranes were as previously described [9]. The anti-PPV CP monoclonal antibodies (mAbs) 3C6 and XR4 were a generous gift of Ingenasa SA. The anti-PPV CP serum was obtained from rabbits immunized with CP obtained from disrupted virions.

3. RESULTS AND DISCUSSION

3.1. *Activity of wild-type NI_a protease on an excess of substrate*

In order to study the capacity of the PPV NI_a protease to cleave an excess of natural or artificial sub-

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strates, we developed a binary *E. coli* expression system, with the sequences encoding the protease and the natural NI_b-CP substrate cloned in the medium copy number plasmid pSU8, and natural and artificial NI_b-CP cleavage site sequences cloned in the high copy number plasmid pUC19. The copy number of the pUC19 derivatives was approximately 5 times higher than that of the pSU8 ones (data not shown). The artificial site consisted in an heptapeptide representing positions -6 to $+1$ at the NI_b-CP junction, inserted in the CP [9]. The proteolytic activity was determined by immunodetection in Western blots of CP-derived peptides. Both the CP derived from processing of the NI_a protease-containing polyprotein encoded by the medium copy number plasmid pPPVS20 and the intact or processed competitor peptides encoded by the high copy number plasmids of the pPPV30 series were recognized by anti-CP polyclonal Ab (figs 1A and 3A) and mAb 3C6 (fig.2A). However, the mAb XR4 only reacted with the CP encoded by pPPVS20 but not with the peptides encoded by pPPV30 series, which lack the carboxy terminal 81 aa of the CP (figs 1B, 2B and 3B).

In cells containing only pPPVS20, efficient processing liberating free CP (36.6 kDa) took place (fig.1A, lane a). As expected, a nonprocessed polypeptide was detected in cells containing plasmids of the pPPV30 series, which do not contain the protease cistron (fig.1A, lanes d, f, h and j). The efficiency of cleavage at the NI_b-CP site encoded by pPPVS20 in the presence of PPV sequences expressed from another plasmid was visualized by immunoreaction with mAb XR4 (fig.1B) which specifically recognized CP sequences from the former plasmid (see above). Similar efficiencies were observed in cells harboring, besides pPPVS20, a high copy number pUC19 derivative (fig.1A and B). The derivatives tested contained either no PPV sequence (pUC19), or the intact (pPPV30 and pPPV30sP16) or mutated (pPPV30Δcp and pPPV30mP5) natural NI_b-CP cleavage site. pPPV30mP5 and pPPV30sP16 contained additional inserted artificial cleavage sites. The activity of the protease encoded by pPPVS20 was enough to cleave the excess wild-type NI_b-CP sites as shown by the disappearance of the intact peptide encoded by pPPV30 and pPPV30sP16, with electrophoretic

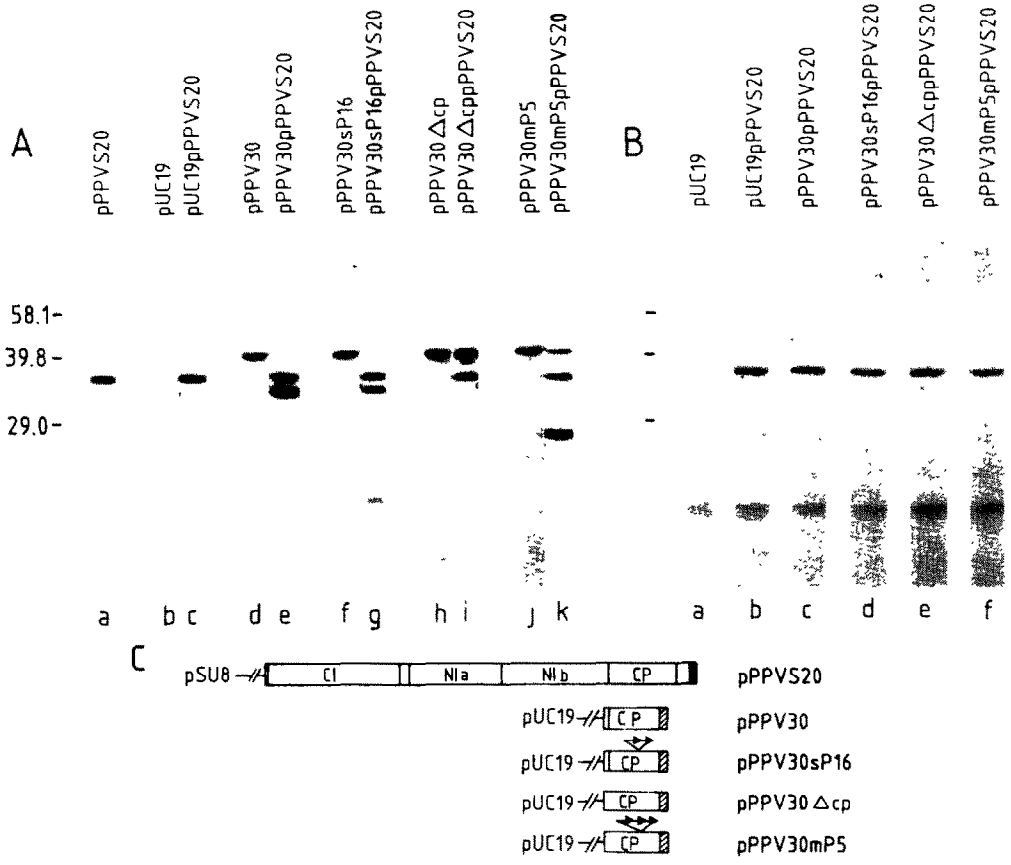

Fig.1. (A and B) Immunoblot of extracts of cells harboring the indicated plasmids, probed with polyclonal anti-PPV CP serum (A) or anti-PPV CP mAb XR4 (B). The molecular masses of biotinylated protein markers are indicated beside the panels. (C) Cistron map of the PPV sequence contained in the different plasmids. The black and hatched boxes represent the poly A tract and the pUC19 sequence encoding the 48 aa fused to the PPV CP cistron, respectively. The 7-aa NI_b-CP artificial sites inserted in the CP [9] are indicated by horizontal arrows. pPPV30Δcp and pPPV30mP5 contain a Gln → Ser substitution at position -1 of the NI_b-CP junction which inhibits proteolytic cleavage at this site [7].

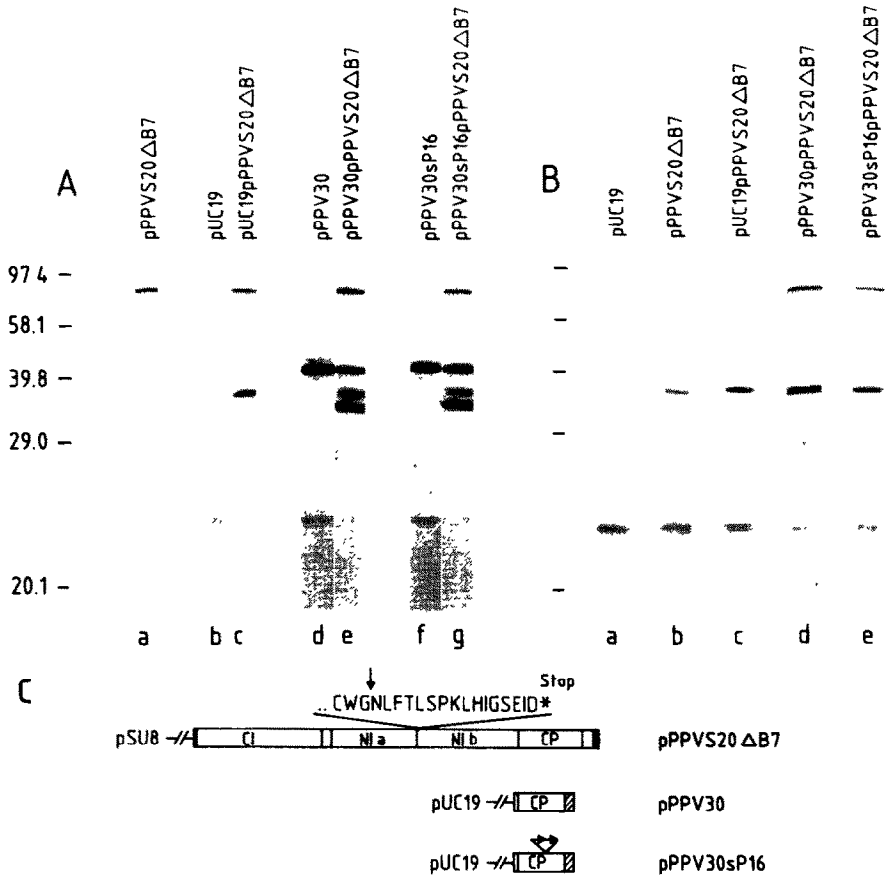

Fig.2. (A and B) Immunoblot of extracts of cells harboring the indicated plasmids, probed with anti-PPV CP mAbs 3C6 (A) or XR4 (B). The molecular masses of biotinylated protein markers are indicated beside the panels. (C) Cistron map of the PPV sequence contained in the different plasmids. The black and hatched boxes and the horizontal arrows indicate the same as in fig.1. The amino acid sequence modified by the ΔB7 mutation is shown above the map. The vertical arrow specifies the end of the wild-type sequence.

mobilities of approximately 40 kDa, and the presence of processed peptides migrating slightly faster than the PPV CP in the extracts containing these plasmids together with pPPVS20 (fig.1A, lanes d–g). These processed peptides were absent in the extracts of cells containing pPPV30Δcp or pPPV30mP5 in addition to pPPVS20 (fig.1A, lanes h–k), indicating that no processing took place at the mutated NI_b-CP site. Further processing at the artificial cleavage sites occurred in the cells harboring pPPV30sP16 and pPPV30mP5 (lanes g and k and see below).

Despite the high activity shown by the protease encoded by pPPVS20 on the natural substrate, the efficiency of processing at the artificial cleavage sites was lower. Processing at the artificial site should produce an amino peptide of 20.7 kDa, which by cleavage at the natural NI_b-CP site would give rise to a 16.3 kDa peptide, and a carboxyl peptide of 16.9 kDa containing sequences of the PPV CP and of pUC19. Indeed, extracts of cells harboring, besides pPPVS20, plasmids encoding in addition to wild-type or mutated natural cleavage sites, also artificial cleavage sites (pPPV30sP16 and pPPV30mP5) contained additional smaller peptides with electrophoretic mobilities corresponding to approximately 23 kDa (pPPV30sP16) or 27 kDa (pPPV30mP5). They also showed bands corresponding to the intact peptide (pPPV30mP5, with a mutated natural NI_b-CP site) or to the peptide processed only at the natural NI_b-CP site (pPPV30sP16) but the intensity of these bands was clearly lower when compared with those of extracts containing plasmids not encoding artificial cleavage sites (pPPV30Δcp and pPPV30) (fig.1A, compare lanes e and g, and i and k).

Although the electrophoretic mobilities of the small peptides were somewhat slower than those expected, their absence in cells harboring plasmids not encoding artificial sites and the different mobilities of the peptides from pPPV30mP5 and pPPV30sP16, in good agreement with the absence or presence of active natural cleavage site, strongly suggest that these small peptides correspond to the amino fragments originating from cleavage at the artificial sites. The carboxy terminal fragment, containing sequences from PPV CP and pUC19 β-galactosidase, was not detected, probably because it was not recognized by the Abs. No quantitative estimation could be made because of the multi-

ple factors affecting the intensity of the bands in Western blots (efficiency of transfer, binding stability, Ab affinity, ...).

3.2. *Activity of the ΔB7 NI_a protease mutant on an excess of substrate*

The activity of the NI_a protease carrying the ΔB7 mutation has been described to be slightly lower than that of the wild-type [7]. The mutation consists in a deletion in the carboxy terminal region of the NI_a cistron producing a frameshift which gives rise to the substitution of several aa and the introduction of a termination codon [7]. For this reason, NI_a ΔB7 and CP sequences are not translated in the same polyprotein; however, internal initiation in the NI_b cistron generated polypeptides containing CP sequences, which were susceptible to cleavage in trans by the protease [7].

In cells containing pPPVS20ΔB7 not only free CP but also the internally initiated 73 kDa peptide were detected (fig.2A, lane a, and B, lane b), indicating that the efficiency of the mutant protease is lower than that of the wild-type. Similar results were obtained when the cells, besides pPPVS20ΔB7, contained pUC19 (lane c), pPPV30 (lane e) or pPPV30sP16 (lane g). No inhibition of the partial cleavage at the NI_b-CP site encoded by pPPVS20ΔB7 took place by competition with the excess of natural NI_b-CP site encoded by pPPV30 or of natural and artificial sites encoded by pPPV30sP16 (fig.2A, lanes c, e and g, and B, lanes c–e), indicating that its efficiency was independent of the amount of substrate. The proportion of the internally initiated peptide and the free CP derived from processing of this peptide was variable, probably related to different amounts of the mutant protease (because the copy number of pPPVS20ΔB7 was not the same in all the samples). In any case, no correlation could be observed between excess of competitor cleavage sites and efficiency of processing.

The PPV peptides encoded by pPPV30 and pPPV30sP16 were processed by the mutant protease encoded by pPPVS20ΔB7 with similar efficiency as the internally initiated 73 kDa peptide encoded by this last plasmid (fig.2A, lanes d–g). However, the artificial cleavage sites encoded by pPPV30sP16 were not significantly recognized by the ΔB7 mutant protease. This result supports the previous suggestion [8,9] that in addition to the conserved heptapeptide other sequences could have an effect on substrate activity. Such sequences would not be very relevant for the wild-type protease but would be needed for the weaker activity of the ΔB7 mutant protein.

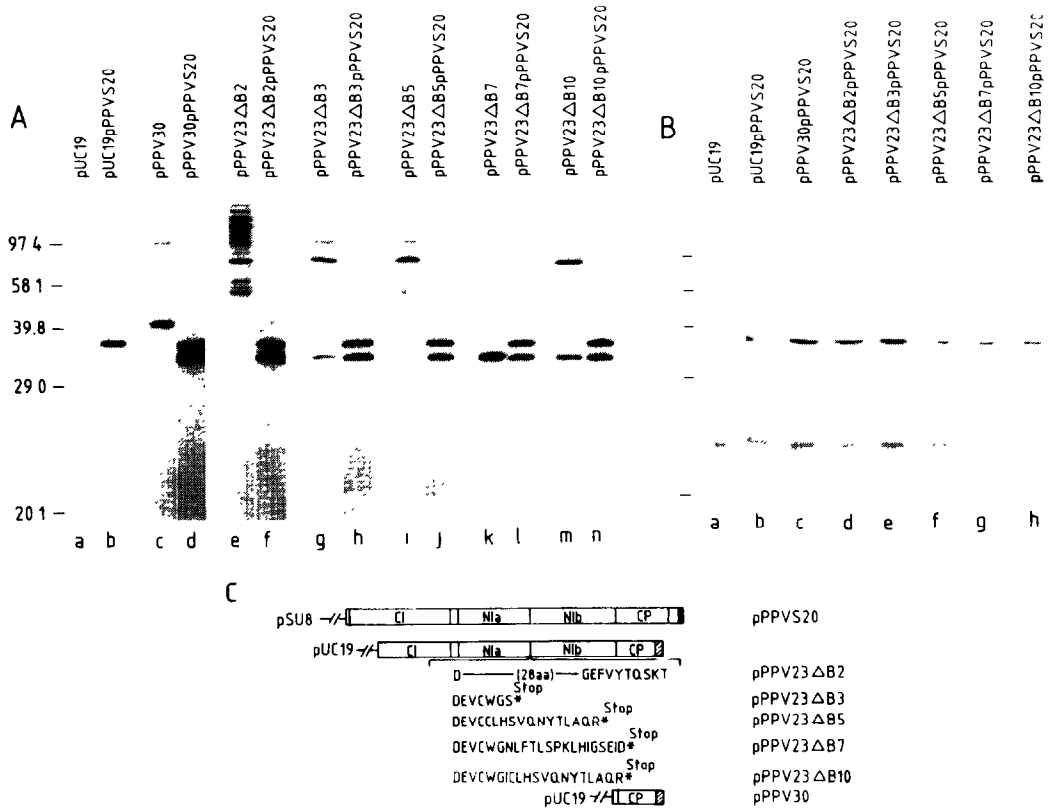

Fig.3. (A and B) Immunoblot of extracts of cells harboring the indicated plasmids, probed with polyclonal anti-PPV CP serum (A) or anti-PPV CP mAb XR4 (B). The molecular masses of biotinylated protein markers are indicated beside the panels. (C) Cistron map of the PPV sequence contained in the different plasmids. The black and hatched boxes indicate the same as in fig.1. The amino acid sequences modified by the deletions of the different plasmids [7] are shown below the map. The number in parenthesis indicates amino acids deleted.

3.3. *Activity of NI_a protein in the presence of excess mutant proteases*

Recently the design of mutant enzymes able to inhibit in vivo the activity of the wild-type proteins has been shown to be a useful approach for interference with viral infection [13]. We have previously described several deletion/substitution mutations at the carboxyl terminus of the NI_a protease which showed different levels of activity [7]. Their ability to compete with the wild-type protease has now been studied using the binary *E. coli* expression system (fig.3). The wild-type NI_a protein was encoded by the medium copy number plasmid pPPVS20 and the competitor mutants by the high copy number plasmids of the pPPV23 series (fig.3C).

The activity of mutant proteases encoded by plasmids with frameshift deletions introducing termination codons (ΔB3, ΔB5, ΔB7 and ΔB10 [7]) was determined by immunodetection of CP liberated by cleavage at the NI_b-CP site of proteins internally initiated at the NI_b cistron (mutants ΔB3, ΔB5, ΔB7 and ΔB10, with out of frame deletions introducing termination codons [7]). The ΔB7 mutant cleaved very efficiently the internally initiated peptides; the activity of the ΔB7 mutant when encoded by the high copy number pPPV23ΔB7 was clearly higher than its activity when encoded by the medium copy number pPPVS20ΔB7 (compare fig.2A, lane a, and B, lane b with fig.3A, lane k), suggesting that, as was commented earlier, its activity is much more dependent on the amount of enzyme than on the substrate concentration. The rest of the mutants showed different proportions of processed/nonprocessed products, the processed band of ΔB2 being undetectable (fig.3A, lanes e, g, i and m). The confused protein pattern in cells containing pPPV23ΔB2 (with a deletion which does not modify the phase of the ORF, lane e) was similar to that described for pPPV12ΔB2 [7] and has not been further analyzed; we cannot discard proteolytic activity of the ΔB2 mutant at other cleavage sites but the absence of processing at the NI_b-CP site strongly argues against this possibility, and probably the multiple bands originate from different internal initiations and nonspecific proteolysis.

In cells containing two plasmids, the wild-type protease encoded by pPPVS20 fully processed the NI_b-CP site-containing peptides encoded by the different pPPV23 plasmids (fig.3A, lanes f, h, j, l and n). The immunoreaction with anti-PPV CP polyclonal Ab (fig.3A) or mAb XR4 (fig.3B) showed that the efficiency of cleavage at the NI_b-CP site from pPPVS20 was mainly not affected by the expression of any of the protease mutants, nor was it affected by the presence of the peptide encoded by pPPV30, which does not contain NI_a protease sequences. We do not think that the lack of competition is due to the fact that the sequences of the NI_a mutants could be forming part of a polyprotein since it has been reported that the NI_a protease does not need to be free to be active [7,14]. Therefore, the inability of the analyzed mutants to outcompete the wild-type protein may indicate that the mutated carboxy terminal region of the protease is involved in the interaction with the substrate.

In summary, the PPV NI_a protease seems to be highly efficient permitting processing of a large excess of substrate. Therefore, the possibility to inhibit its activity by increasing the amount of substrate seems unlikely. New approaches will require the characterization of the proteolytic active site and critical domains involved in the substrate-enzyme interaction in order to design artificial cleavage sites with higher affinity than the natural sites or mutant proteins devoid of enzymatic activity but able to strongly interact with the substrate.

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