Expression and partial purification of recombinant tomato ringspot nepovirus 3C-like proteinase: comparison of the activity of the mature proteinase and the VPg-proteinase precursor

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Abstract

The 3C-like proteinase (Pro) from *Tomato ringspot virus* (genus *Nepovirus*) is responsible for the processing of the RNA1-encoded (P1) and RNA2-encoded (P2) polyproteins. Cleavage between the VPg and Pro domains is inefficient in vitro and in *E. coli*, resulting in the accumulation of the VPg-Pro. In this study, we have compared the *trans*-activity of the Pro and VPg-Pro on various P1- and P2-derived precursors. Recombinant Pro and VPg-Pro were partially purified using an *E. coli* expression system. A mutation of the VPg-Pro cleavage site was introduced into the VPg-Pro to prevent slow release of the Pro. The Pro was five to ten times more active than the VPg-Pro on two P2 cleavage sites (at the N- and C-termini of the movement protein domain) and was approximately two times more active than the VPg-Pro on the third P2 cleavage site (between the X3 and X4 domains). Neither the Pro nor the VPg-Pro could cleave in *trans* P1-derived substrates containing the three cleavage sites delineating the X1, X2, putative NTP-binding protein and VPg domains. These results are discussed in light of the possible regulation of the proteinase activity during virus replication. Crown Copyright © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Proteolytic processing of precursor polyproteins is a key step in the replication cycle of many viruses. It is tightly regulated, allowing step-wise release of precursor and mature forms of the viral proteins (for reviews see Dougherty and Semler, 1993; Spall et al., 1997). The picornavirus 3C proteinases (3C-Pro) and the related 3C-like proteinases from coronaviruses, potyviruses, comoviruses and nepoviruses are a well-studied group of enzymes that have structural homology to chymotrypsin (Bazan and Fletterick, 1988; Gorbalenya et al., 1989; Allaire et al., 1994;

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Matthews et al., 1994; Mosimann et al., 1997). Proteolytic processing efficiency at different 3C-Pro and 3C-like-Pro cleavage sites in the viral polyproteins can vary widely. Predominant cleavage sites are generally characterized by a conserved amino acid sequence at the cleavage site (Dougherty et al., 1988; Long et al., 1989; Pallai et al., 1989) and by favorable secondary structure of the protein around the cleavage site (Ypma-Wong et al., 1988b). Additional mechanisms also contribute to the regulation of the proteinase activity at specific cleavage sites. In comoviruses, a co-factor increases the activity of the proteinase on some cleavage sites but decreases its activity on other sites (Peters et al., 1992a,b). In poliovirus, the activity of the 3C-Pro is regulated by the presence of the 3D domain on intermediate precursors (Ypma-Wong et al., 1988a; Jore et al., 1988) and by the interaction of the 3C-Pro with the 3AB protein (Molla et al., 1994). In two nepoviruses of subgroups A and B, the presence of the VPg domain on partially processed precursors of the proteinase was shown to modulate the activity of the proteinase although the specific effect of the VPg domain on the proteinase activity differed between the two viruses considered (Margis et al., 1994; Hemmer et al., 1995).

Tomato ringspot virus (ToRSV) is the only member of the subgroup C of nepoviruses for which the proteolytic processing of the RNA1- and RNA2-encoded polyproteins (P1 and P2, respectively) has been extensively studied. The cleavage site specificity of the ToRSV proteinase was shown to be different from that of proteinases of nepoviruses of subgroups A and B (Hans and Sanfaçon, 1995; Carrier et al., 1999) and a consensus amino acid sequence for ToRSV cleavage sites was identified [(C or V)Q/(G or S); Wang et al., 1999; Carrier et al., 1999]. Mutational analysis of two ToRSV cleavage sites has confirmed the importance of these amino acids in processing efficiency (Carrier et al., 1999). Also, cleavage sites were identified at the N-terminus of each of the ToRSV polyproteins which are not present in the polyproteins of nepoviruses of subgroups A and B (Wang and Sanfaçon, 2000; Carrier et al., 2001). Indeed, the ToRSV P1 polyprotein is cleaved at five cleavage sites and

the P2 polyprotein is cleaved at three cleavage sites (instead of four and two cleavage sites in the P1 and P2 polyproteins, respectively, of characterized nepoviruses of subgroups A and B).

Using an expression vector containing the region of RNA1 coding for the VPg, the proteinase and a small region of the polymerase, we have earlier shown proteolysis at the Pro-Pol and at the VPg-Pro cleavage sites in *E. coli* (Wang et al., 1999). Cleavage at the VPg-Pro cleavage site was inefficient in a variety of polyprotein precursors produced in an *E. coli* expression system or in an vitro translation system, resulting in the accumulation of the VPg-Pro precursor (Wang et al., 1999; Wang and Sanfaçon, 2000). This suggested that the VPg-Pro precursor may play a special role in the regulation of the processing of ToRSV polyproteins. Therefore it was of interest to determine the activity of the mature ToRSV proteinase in comparison to that of the VPg-Pro precursor. Here we report the purification of active recombinant ToRSV proteinase from *E. coli* cells in its mature (Pro) and precursor (VPg-Pro) forms and the characterization of their proteolytic activities.

2. Materials and methods

2.1. Plasmid construction

To allow expression of the mature Pro and of the VPg-Pro precursor in *E. coli*, plasmids pET21d-Pro and pET21d-VPg-Pro were constructed. To prepare the plasmid pET21d-Pro, an intermediate plasmid pET21d-Pro-N-Pol was constructed by inserting the *Nco*I-*Eco*RI fragment from pET15b-Pro-N-Pol (Wang et al., 1999) into the corresponding sites of the vector pET21d (Novagen). Plasmid pET21d-Pro was derived from the intermediate plasmid by performing in vitro mutagenesis (Fisher and Pei, 1997) using primers JC-1 (5′ CACCACCACCACCACCACTGAG 3′, from the pET21d vector sequence, Novagen) and 39 (5′ CTGGCAAGGAGCAAAAGAAG 3′, corresponding to nts 4535–4515 on ToRSV RNA1, numbering according to Rott et al., 1995) and Pfu polymerase (Stratagene). This removed the DNA sequence corresponding to the N-terminal region

of the polymerase gene and introduced the His(x6) tag in frame with the C-terminus of the proteinase. Plasmid pET21d-VPg-Pro was derived from plasmid pET15b-VPg-Pro-N-Pol (Wang et al., 1999) in a similar manner using primers JC-1 and 39 as above. To introduce a mutation in the VPg-Pro cleavage site (substitution of the Gln at the -1 position of the cleavage site for an Ala) encoded by plasmids pET21d-VPg-Pro and pET15b-VPg-Pro-N-Pol, site-directed mutagenesis was conducted using primers 53 (5′ ***CGC***TACA-GATTGTGGGCGGAA 3′; nts 3794–3774 of RNA1, altered sequence is bold and italicized) and 52 (5′ GGTTCTTCTTTGGCGGAAGCG 3′, complementary to nts 3795–3816 on RNA1) to produce plasmids pET21d-VPg-Pro^{Q1264A} and pET15b-VPg- Pro-N-Pol^{Q1264A}, respectively. To construct plasmid pT7-cX1-X2, a 1151 bp fragment of a full-length clone of RNA1 (plasmid pMR10, Rott et al., 1995) was amplified using Pfu polymerase (Stratagene) and earlier described oligonucleotides W034 (corresponding to RNA1 nts 786–801 and engineered to include a *Nco*I site; Wang and Sanfaçon, 2000) and W027 (complementary to RNA1 nts 1922–1937 and engineered to include a *Sal*I site). The amplified fragment was digested with *Nco*I and *Sal*I and inserted in the corresponding sites of plasmid pCITE-4a(+) (Novagen). The sequence of all plasmids was verified by automated sequencing (Perkin-Elmer) using an ABI 310 Genetic Analyzer.

2.2. Expression and purification of fusion proteins

Plasmids pET21d-Pro and pET21d-VPg-Pro^{Q1264A} were transformed into *E. coli* BL21(DE3) cells as outlined in the supplier's protocol (Novagen). To purify the expressed proteinases from the soluble fraction, BL21(DE3) cells harboring plasmids pET21d-Pro and pET21d-VPg-Pro^{Q1264A} were grown to an OD$_{600}$ of 0.5 and induced with 1 mM isopropyl-thio-β-galactoside (IPTG) for 5 h at 25 °C. Cells were collected by centrifugation, resuspended in lysis buffer (50 mM NaH$_2$PO$_4$, pH 8.0; 0.3 M NaCl; 10 mM imidazole) and lyzed using a French press. The extracts were centrifuged and the supernatant

adjusted to 10 mM 2-mercaptoethanol and 0.1% Tween-20. Precharged Ni^{+2} nitrilotriacetic acid (NTA)-agarose (0.1 ml of matrix pre-equilibrated in lysis buffer) was added and the mixture incubated with gentle agitation for 90 min at 4 °C. The suspension was then loaded into a column and washed with lysis buffer plus 10% glycerol containing 20 mM imidazole and then 50 mM imidazole. Finally, proteinases were eluted using 250 mM imidazole in lysis buffer (without glycerol) and dialyzed against 50 mM Tris–HCl, pH 7.6, 0.3 M NaCl and 1 mM DTT. Proteinase preparations were stored in aliquots in the dialysis buffer plus 10% glycerol at -70 °C.

The relative concentration of Pro and VPg-Pro in the preparations was estimated using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as a standard. SDS-PAGE analysis (Laemmli, 1970) followed by quantitation of band densities using ImageQuant (Molecular Dynamics) was used to normalize the amount of Pro and VPg-Pro taken for activity assays.

2.3. In vitro translations and activity assays

The MP-CAT, X4-MP, X3-X4, cX1-X2 and cNTB-VPg substrates were synthesized in vitro using the coupled TNT transcription/translation rabbit reticulocyte system (Promega) in the presence of [^{35}S]-methionine (New England Nuclear) with the plasmids pET-MP-CAT (Wang and Sanfaçon, 2000), pT3-X-MP (Carrier et al., 1999), pT7-N-TermFL (for the X3-X4 substrate, Carrier et al., 2001), pT7-cX1-X2 and pT7-cNTB-VPg (Wang and Sanfaçon, 2000) as earlier described (Wang and Sanfaçon, 2000). To produce substrate X2-NTB, plasmid pT7-X2-Pro (Wang and Sanfaçon, 2000) was digested with *Xho*I (located at nt 3393 on ToRSV RNA1), and run-off transcripts were synthesized in vitro using the T7 RNA polymerase (Gibco-BRL) as described earlier (Hans and Sanfaçon, 1995). The run-off transcript was then used as a template in the standard rabbit reticulocyte system (Promega) as described earlier (Hans and Sanfaçon, 1995). Proteolytic processing of the substrates was tested by adding exogenous proteinase to the in vitro translation products. Proteolytic processing reactions were performed

in the presence of 1 mM DTT; 100 mM Tris–HCl, pH 7.5 and 10% glycerol. The reactions were stopped by the addition of one volume of (2×) SDS-PAGE sample buffer (120 mM Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol; 0.02% bromophenol blue) and analyzed by SDS-PAGE. Time-course experiments were conducted using several dilutions of the proteinase to ensure that the reactions were in the linear range. The gels were scanned using a PhosphorImager (Molecular Dynamics). The amount of radioactivity in individual bands was measured using ImageQuant as described by the supplier. The cpm values were adjusted for the number of methionine residues present in each protein. The percentage of precursor that was converted to one of the cleaved products (32 kDa MP for the MP-CAT precursor, 38 kDa MP for the X4-MP precursors and 38.5 kDa X3 for the X3-X4 precursor) was calculated at each time point as earlier described in Carrier et al. (1999). The cleavage products chosen for this calculation met the following two criteria: the product was stable over time and it migrated in an area of the gel devoid of contaminating background bands. Very similar results were obtained when the calculation was made with the other cleavage product (data not shown). In the case of the X4-MP precursor only the predominant precursor is considered in the calculation. As earlier mentioned (Carrier et al., 1999), other minor precursors were also produced by internal translation initiation and similar processing kinetics were obtained when these precursors were also considered in the calculation (data not shown). In experiments, where the effect of temperature or pH on the activity of the Pro and VPg-Pro was tested, the proteinases were first preincubated at the appropriate temperature or in the appropriate buffer for 30 min prior to the addition of the MP-CAT substrate.

2.4. Immunodetection of recombinant proteinase

For Western blotting, proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to PVDF membranes (Bio-Rad). The filters were blocked using skim milk powder and probed with monoclonal antibodies raised against the proteinase or polyclonal antibodies raised against the VPg as earlier described (Wang et al., 1999).

3. Results

3.1. Purification of recombinant ToRSV proteinase and VPg-proteinase

To obtain purified recombinant proteinase the corresponding region of the ToRSV RNA1 cDNA was introduced into the expression vector pET21d (Novagen). As the cleavage sites between the Pro and Pol and between the VPg and Pro have been determined earlier (Wang et al., 1999), the coding region for the Pro or the VPg-Pro was excised precisely and inserted into pET21d as described in Section 2 to give plasmids pET21d-Pro and pET21d-VPg-Pro (Fig. 1). To allow affinity purification of the recombinant proteins, a histidine tail was introduced immediately after the last amino acid at the C-terminus of the proteinase domain.
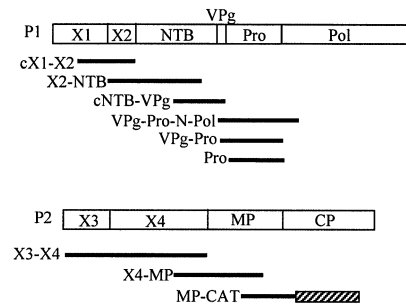

Fig. 1. Schematic representation of RNA1 and RNA2-derived substrates and proteinases. The RNA1- and RNA2-encoded polyproteins (P1 and P2, respectively) are shown with the boxes. Vertical lines within these boxes represent earlier identified cleavage sites. The portion of the viral polyprotein included in the different precursors is shown by the thick lines while the hatched box line in the MP-CAT precursor represents the chloramphenicol acetyl transferase reporter protein which is fused in frame with the viral proteins. The functions of the different viral proteins are abbreviated as follows: NTB, putative NTP-binding protein; VPg, genome-linked viral protein; Pro, proteinase; Pol, RNA-dependent RNA polymerase; MP, movement protein; CP, coat protein. The functions of the X1, X2, X3 and X4 proteins are not known.
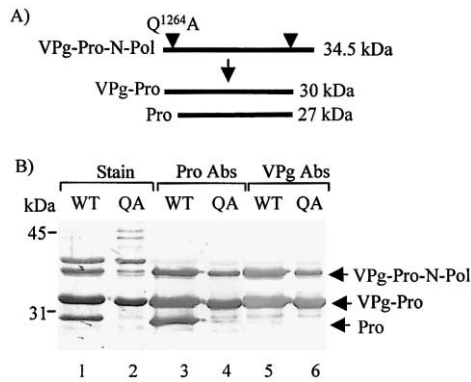
A)

B)

Fig. 2. Cleavage at the VPg-Pro site is abolished by a point mutation (Q^{1264}A) at the -1 position of the cleavage sites. (A) Schematic representation of the VPg-Pro-N-Pol precursor used to study the effect of a point mutation in the VPg-Pro cleavage site. The positions of the VPg-Pro and Pro-Pol cleavage sites on the precursor are shown by the arrowheads. The RNA1-encoded polyprotein (P1) from which the precursor was derived is shown at the top of the figure. (B) Cis-processing of the VPg-Pro-N-Pol wild-type and mutated precursor in induced E. coli cells. E. coli BL21 (DE3) containing either the wild-type pET15b-VPg-Pro-N-Pol (WT) (Wang et al., 1999) or the mutated pET15b-VPg-Pro-N-Pol^{Q1264A} (QA) plasmids were induced with IPTG at 25 °C. Inclusion bodies were purified 3 h after induction and proteins present in these preparations were separated by SDS-PAGE (12% polyacrylamide). Proteins were transferred to PVDF membranes and stained with Coomassie Brilliant Blue (Stain), or immunodetected with monoclonal antibodies against the proteinase (Pro Abs) or with polyclonal antibodies against the VPg (VPg Abs). The positions of the VPg-Pro-N-Pol precursor and of the VPg-Pro and Pro cleaved products are indicated on the right side of the gel. Migration of molecular weight standards is indicated on the left side of the gel.

Preliminary results using plasmid pET21d-VPg-Pro revealed that slow processing of the VPg-Pro precursor occurred at the VPg-Pro cleavage site when expression was performed at low temperatures (25 °C) allowing the release of small amounts of the mature Pro (data not shown). Slow processing at the VPg-Pro cleavage site of a larger precursor (pET15b-VPg-Pro-N-Pol, see Fig. 1 and Fig. 2A) containing the N-terminal region of the polymerase was also earlier shown (Wang et al., 1999). To eliminate this processing and ensure that the VPg-Pro preparation did not contain trace amounts of the mature proteinase, a mutation was introduced at the -1 position of

the VPg-Pro cleavage site (Q^{1264} to A). To verify that this mutation did not negatively affect the proteolytic activity of the Pro, this mutation was first introduced into the VPg-Pro-N-Pol precursors and cis-cleavage at the Pro-Pol site was monitored. Expression of the wild-type VPg-Pro-N-Pol precursor at 25 °C resulted in the release of the VPg-Pro intermediate and of the mature proteinase as earlier described (Wang et al., 1999 see Fig. 2B, lanes 1, 3 and 5). Following expression of the VPg-Pro-Pol^{Q1264A} precursor at 25 °C, the VPg-Pro cleavage product was detected but not the mature proteinase (see Fig. 2B, lanes 2, 4 and 6). This result indicated that the Q^{1264} to A mutation prevented cleavage at the VPg-Pro site and further that it did not affect the proteolytic activity of the proteinase on the Pro-Pol site. This mutation was, therefore, introduced into the VPg-Pro precursors to allow the production of recombinant ToRSV VPg-Pro.

The Pro and VPg-Pro (containing the Q^{1264} to A mutation) fusion proteins were expressed in E. coli at different temperatures. Under all temperatures tested, most of the expressed protein was found in the insoluble fraction of the extract from induced bacterial cells (data not shown). However, at lower temperatures (25 °C), some protein remained in the soluble fraction. Recombinant proteinase was purified from the soluble fraction as described in Section 2. The degree of purity of the proteinase obtained by this method was 80–90% after purification using Ni^{+2}-NTA-agarose (as estimated by SDS-PAGE analysis, see Fig. 3) and the yield was 50–100 μg of purified
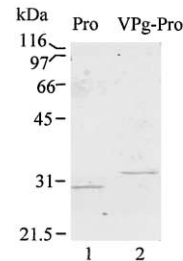

Fig. 3. Purification of recombinant ToRSV Pro and VPg-Pro. Partially purified preparations of recombinant Pro and VPg-Pro were separated by SDS-PAGE (12% polyacrylamide) and stained with Coomassie Brilliant Blue.

proteinase per liter of culture. Preparations of recombinant proteinase were tested for their proteolytic activity using the earlier described MP-CAT substrate which contains the MP-CP cleavage site (Wang and Sanfaçon, 2000). As shown earlier, incubation of the MP-CAT precursor with the recombinant proteinase resulted in the production of two additional proteins that corresponded to the cleaved MP and the CP-CAT proteins (see below). Mutated recombinant proteinases (i.e. containing a His to Asp substitution in the putative catalytic triad or a His to Leu substitution in the putative substrate binding pocket) purified using the same method were not active on the MP-CAT substrate (data not shown). This demonstrated that the activity observed with the wild-type recombinant proteinase was not due to an *E. coli* protease contaminating the preparation. Recombinant proteinase was also purified from the insoluble fraction and resolubilized by progressive dialysis in decreasing concentration of urea. Although highly purified proteinase was obtained by this method, the specific activity of the proteinase was ten to a hundred times less than that obtained with the proteinase purified from the soluble fraction suggesting that only a portion of the molecules were properly refolded (data not shown). Further experiments were conducted using proteinase preparations obtained from the soluble fraction.

The optimum temperature and pH for activity of the ToRSV Pro and VPg-Pro were tested using the MP-CAT substrate (Fig. 4). Relatively efficient cleavage was evident at temperatures examined from 16 to 26 °C (with an optimum around 21 °C) but diminished significantly at temperatures outside this range. Finally, optimum pH for activity of the Pro and VPg-Pro was determined to be between pH 7 and 8. Further experiments were conducted at 21 °C and at pH 7.5.

3.2. Differential activity of the Pro and VPg-Pro on RNA-2 derived substrates

The *trans*-processing activities of the Pro and VPg-Pro (containing the Q^{1264} to A mutation) were compared on several RNA-2 substrates. As a first step, precursor MP-CAT containing the MP-
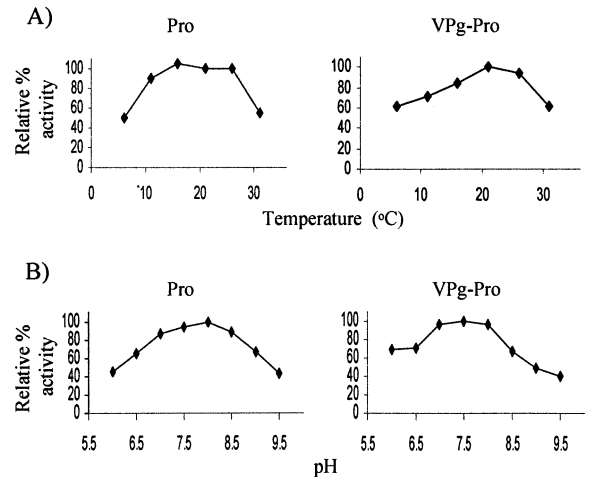

Fig. 4. Effect of the temperature and pH on the proteolytic activity of the Pro and VPg-Pro on the MP-CAT substrate. (A) Proteolytic assays were performed in 100 mM sodium phosphate buffer, 1 mM DTT and 10% glycerol at 6, 11, 16, 21, 26 and 31 °C using the MP-CAT substrate. Proteolytic activity under the different conditions was measured as described in Section 2. The activity at each temperature is expressed as a percentage of the activity measured at 21 °C. The relative percentage of activity under each condition represents an average of at least three independent experiments. (B) Proteolytic assays were performed at 21 °C either in 100 mM phosphate buffer adjusted to pH 6, 6.5, 7, 7.5, 8 and 8.5 or in 100 mM Tris–HCl adjusted to pH 7.5, 8, 8.5, 9 and 9.5. The activity under each condition is expressed as a percentage of the activity measured at pH 7.5 with the appropriate buffer. As in (A), the relative percentage of activity under each condition represents an average of at least three independent experiments.

CP cleavage site was tested (Fig. 5A). The molecular masses of the Pro and VPg-Pro differed by only 10% (27 kDa for Pro and 30 kDa for VPg-Pro). Therefore, equivalent amounts of each proteinase (in ng) were initially used to compare their activities. The Pro was found to be considerably more active than the VPg-Pro on the MP-CAT substrate (Fig. 5B). To determine more precisely the relative processing activities of the Pro and VPg-Pro, time-course experiments were conducted on serial dilutions of several individual proteinase preparations to ensure that the reactions were in the linear range. A representative experiment is shown in Fig. 5C and D. The results revealed that the Pro was 5–10 times more active than the VPg-Pro on the MP-CAT substrate.
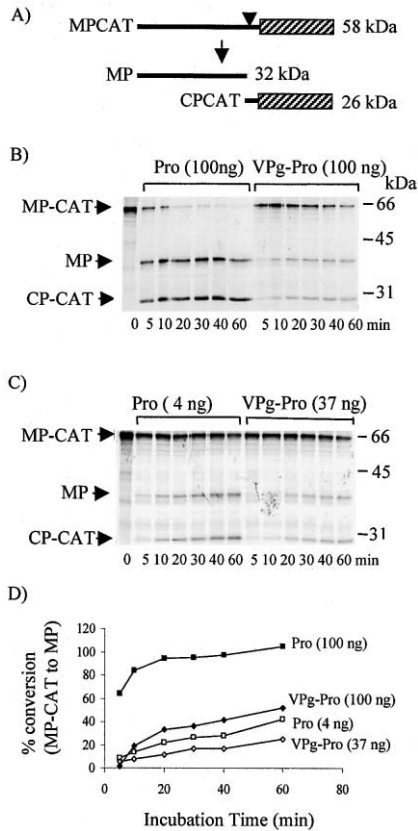
Fig. 5. Comparison of the proteolytic activities of the Pro and VPg-Pro on the MP-CAT substrates. (A) Schematic representation of the proteolytic processing of the MP-CAT precursor. Position of the cleavage site on the precursor is shown by the arrowhead. The calculated molecular masses of the MP-CAT precursor and of the two cleavage products (MP and CP-CAT) are shown. Viral sequences are shown by the continuous black lines, and sequence from the CAT protein (fused in-frame with the viral sequences) is shown by the hatched boxes. (B) Time-course experiment of the processing of the MP-CAT substrate by equivalent amounts of the purified recombinant Pro and VPg-Pro (VPg-Pro^{Q1264A}). The MP-CAT substrate and the cleaved products were separated by SDS-PAGE (12% polyacrylamide) and visualized by autoradiography. The position of the precursor and cleaved products are indicated on the left side of the gel, while the positions of molecular mass markers are shown on the right side of the gel. (C) Time-course experiments with adjusted amounts of each proteinase resulting in linear kinetics of cleavage of the MP-CAT substrate. (D) Graphical representation of the cleavage kinetics of the MP-CAT substrate using the Pro or the VPg-Pro. The percentage of conversion of the MP-CAT substrate to the MP cleaved product was calculated as described in Section 2.

Proteolytic activities of the Pro and VPg-Pro were also tested on substrates containing the other two P2 cleavage sites. Results with the X4-MP substrate (containing the cleavage site at the N-terminus of the movement protein domain) were similar to those obtained with the MP-CAT substrate (see Fig. 6). Indeed, the Pro was found to be also 5–10 times more active than the VPg-Pro on the X4-MP substrate.

We have earlier shown that in vitro processing of the X3-X4 precursor is considerably less efficient than processing of the X4-MP and MP-CAT precursors (Carrier et al., 2001). Therefore, reactions were conducted using 920 ng of proteinase (compared with 4–100 ng of proteinase used for the MP-CAT and X4-MP substrates) for an extended incubation time. In experiments using equivalent amounts of each proteinase, the Pro was clearly more active on the X3-X4 substrate than the VPg-Pro (Fig. 7). However, the difference between the efficiency of cleavage of the two proteinase was not as pronounced as with the X4-MP and MP-CP cleavage sites. Cleavage of the X3-X4 substrate was approximately two times more efficient with the Pro than with the VPg-Pro.

3.3. The VPg-Proteinase is not active in trans on cleavage sites at the N-terminus of the P1 polyprotein

Our earlier studies have shown that the ToRSV mature proteinase (Pro) was not able to cleave P1 cleavage sites in *trans* (Carrier et al., 1999; Wang and Sanfaçon, 2000). In a related nepovirus (*Grapevine fanleaf virus*, GFLV, a nepovirus of subgroup A), although the mature proteinase could not process P1 cleavage sites in *trans*, the VPg-Pro precursor had the ability to cleave at least one P1 cleavage site in *trans* (X-NTB cleavage site, located immediately upstream of the NTB domain; Margis et al., 1994). To investigate the possibility that the ToRSV VPg-Pro may possess a similar activity, in vitro *trans*-processing assays were conducted with the purified VPg-Pro (Q^{1264} to A). Several precursors were tested which included three earlier described cleavage sites in the N-terminal region of P1 (the X1-X2, X2-NTB
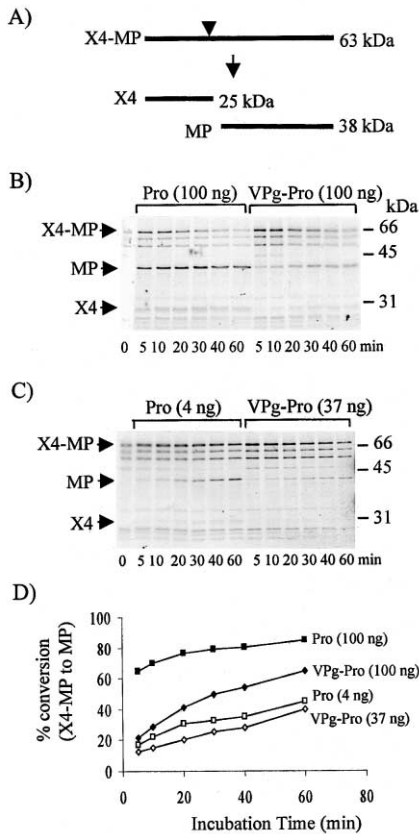
Fig. 6. Comparison of the proteolytic activities of the Pro and VPg-Pro on the X4-MP substrate. (A) Schematic representation of the proteolytic processing of the X4-MP precursor. Position of the cleavage site on the precursor is shown by the arrowhead. The calculated molecular masses of the X4-MP precursor and of the two cleavage products (MP and X4) are shown. (B) Time-course experiment of the processing of the X4-MP substrate by equivalent amounts of the purified recombinant Pro and VPg-Pro (VPg-Pro^{Q1264A}). The X4-MP substrate and the cleaved products were separated by SDS-PAGE (12% polyacrylamide) and visualized by autoradiography. The positions of the precursor and cleaved products are indicated on the left side of the gel, while the positions of molecular mass markers are shown on the right side of the gel. (C) Time-course experiments with adjusted amounts of each proteinase resulting in linear kinetics of cleavage of the X4-MP substrate. (D) Graphical representation of the cleavage kinetics of the X4-MP substrate using the Pro or the VPg-Pro. The percentage of conversion of the X4-MP substrate to the MP cleaved product was calculated as described in Section 2.

and NTB-VPg cleavage sites; Wang and San-façon, 2000). The precursors (cX1-X2, X2-NTB, cNTB-VPg) used are shown in Fig. 8A. As a control for the proteinase activity, the X3-X4 precursor was included in this study. To ensure that low levels of cleavage could be detected, the incubation period was extended to 16 h. The reactions were conducted at 16 °C to preserve the activity of the proteinase during the incubation. Although cleavage of the X3-X4 precursor was evident under these conditions, cleavage of the cX1-X2, X2-NTB and cNTB-VPg precursors was not detected (Fig. 8B).
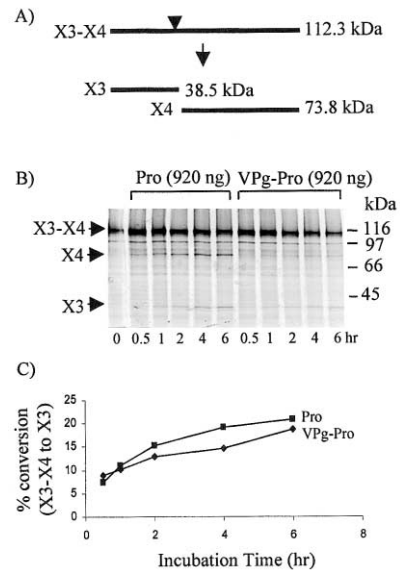

Fig. 7. Comparison of the proteolytic activities of the Pro and VPg-Pro on the X3-X4 substrate. (A) Schematic representation of the proteolytic processing of the X3-X4 precursor. Position of the cleavage site on the precursor is shown by the arrowhead. The calculated molecular masses of the X3-X4 precursor and of the two cleavage products (X3 and X4) are shown. (B) Time-course experiment of the processing of the X3-X4 substrate by the purified recombinant Pro and VPg-Pro (VPg-Pro^{Q1264A}). The X4-MP substrate and the cleaved products were separated by SDS-PAGE (9% polyacrylamide) and visualized by autoradiography. The positions of the precursor and cleaved products are indicated on the left side of the gel, while the positions of molecular mass markers are shown on the right side of the gel. (C) Graphical representation of the cleavage kinetics of the X3-X4 substrate using the Pro or the VPg-Pro. The percentage of conversion of the X3-X4 substrate to the X3 cleaved product was calculated as described in Section 2.
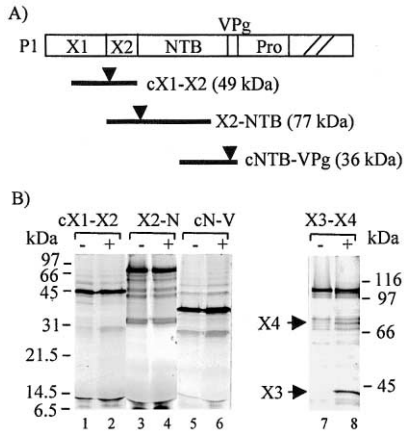
Fig. 8. *Trans*-processing assays of the VPg-Pro on RNA-1 (cX1-X2, X2-NTB, cNTB-VPg) and RNA-2 (X3-X4) derived substrates. (A) Schematic representation of the cX1-X2, X2-NTB and cNTB-VPg substrates. The calculated molecular mass of each substrate is indicated in parenthesis and the position of the cleavage sites on each substrate is indicated by the arrowheads. (B) Assays were conducted at 16 °C for 16 h in the presence (+) or in the absence (−) of recombinant VPg-Pro (300 ng). Processing reactions were separated by SDS-PAGE [15% polyacrylamide for the cX1-X2, X2-NTB (X2-N) and cNTB-VPg (cN-V) substrates (lanes 1–6) and 8% polyacrylamide for the X3-X4 substrate (lanes 7 and 8)]. The positions of the X3 and X4 cleaved products is shown on the left side of the gel. The positions of the molecular weight standards are indicated on the left side of the gel for lanes 1–6 and on the right side of the gel for lanes 7 and 8.

4. Discussion

In this study, we have expressed and partially purified recombinant ToRSV proteinase as a mature protein and as a precursor (VPg-Pro). Although active recombinant 3C or 3C-like proteinases have been obtained for a number of picornaviruses, potyviruses and coronaviruses (see for example Ivanoff et al., 1986; Nicklin et al., 1988; Malcolm et al., 1992; Birch et al., 1995; Ménard et al., 1995; Parks et al., 1995; Hall and Palmenberg, 1996; Seybert et al., 1997), this is the first example of the purification of active recombinant proteinase for a virus of the family *Comoviridae*. The availability of active recombinant ToRSV proteinase will facilitate further studies on its activity and on the design of specific inhibitors of the proteinase.

The optimum temperature for the ToRSV proteinase proteolytic activity on the P2-derived MP-CAT substrate was around 21 °C. Using a P1-derived precursor containing the NTB, VPg and Pro domains, *cis*-processing at the NTB-VPg cleavage site was also shown to be more efficient at temperatures between 16 and 21 °C (Wang, 1998). The preference for lower temperatures was similar to that observed for the *Turnip mosaic virus* (TuMV, genus *Potyvirus*) NIa proteinase (Kim et al., 1996) but differed from that observed for 3C or 3C-like proteinases from animal viruses [for example the *Mengo virus* (genus *Cardiovirus*) 3C proteinase exhibits an optimal temperature of 37 °C, see Hall and Palmenberg, 1996]. This may, therefore, represent an adaptation to the plant hosts, which normally grow at lower temperature than mammalian cells.

In earlier studies, the VPg-Pro was found to accumulate in in vitro assays, and following the expression of various larger precursors in *E. coli* (Wang, 1998; Wang et al., 1999; Wang and Sanfaçon, 2000). Similarly, inefficient cleavage at the VPg-Pro site was observed for two nepoviruses (GFLV and *Tomato black ring virus*, TBRV; Margis et al., 1994; Hemmer et al., 1995) and for two potyviruses (*Tobacco etch virus* and TuMV Dougherty and Parks, 1991; Laliberté et al., 1992) resulting in accumulation of either the VPg-Pro or VPg-Pro-Pol precursors. In this study, we have compared the specific activity of the ToRSV Pro and VPg-Pro. The VPg-Pro was 5–10 times less active than the Pro on precursors containing the X4-MP and MP-CP cleavage sites. In vitro processing at the X3-X4 cleavage site was less efficient, and the Pro was approximately two times more active than the VPg-Pro on this precursor.

We have tested the potential *trans*-activity of the ToRSV proteinase on several P1 cleavage sites (X1-X2, X2-NTB, NTB-VPg). Neither the Pro nor the VPg-Pro could cleave these sites in a *trans*-fashion (Wang and Sanfaçon, 2000; and this study). However, we have earlier shown that the presence of the VPg domain on precursors containing the Pro-Pol cleavage site increased the efficiency of *cis*-cleavage at this site (Wang et al., 1999). Taken together these results suggest that the presence of the VPg domain modulates the

activity of the proteinase by increasing *cis*-cleavage at the Pro-Pol site and decreasing the *trans*-activity of the proteinase on the RNA2-encoded cleavage sites.

We do not know whether the VPg-Pro also accumulates in infected plants. We have attempted the detection of either the Pro or the VPg-Pro in vivo. However, we were unsuccessful in detecting these proteins in total extracts or in membrane-enriched fractions from ToRSV- infected cucumber or *Nicotiana benthamiana* plants. This could be explained by a low concentration of these proteins in the plant or by specific modifications of these proteins in plants that preclude their recognition by the antibodies used in this study. At this point, we can only speculate that the VPg-Pro may be the predominant form of proteinase activity early during the course of infection although we cannot exclude the possibility that plant factors may influence the processing of the P1 polyprotein by increasing cleavage at the VPg-Pro site. Assuming that initial polyprotein processing of P1 in vivo parallels that observed in vitro, the slow release of the mature proteinase from the VPg-Pro precursor may provide a mechanism by which *cis*-cleavage of the P1 polyprotein at the Pro-Pol site is favored early in infection allowing the release of proteins involved in viral replication while *trans*-cleavage of the P2 polyprotein is more efficient later in infection allowing the release of the movement protein and coat protein, and subsequently of the X3 and X4 proteins.

Differential proteolytic activities in precursor and mature forms of the proteinase have also been shown for related nepoviruses. Indeed, the GFLV VPg-Pro was found to be less active than the Pro on RNA2-encoded cleavage sites and more active than the Pro on an RNA1-encoded cleavage site (Margis et al., 1994). However, in contrast with the ToRSV proteinase, the presence of the VPg domain on precursors containing the GFLV proteinase did not influence the *cis*-processing of the Pro-Pol cleavage site but instead, increased *trans*-processing at the cleavage site upstream of the NTB domain. Interestingly, in the case of another nepovirus (TBRV), the VPg domain was also shown to modulate the activity of

the proteinase (Hemmer et al., 1995). The presence of the VPg domain on precursors containing the TBRV proteinase resulted in increased *trans*-cleavage of RNA2-encoded cleavage sites and increased *cis*-cleavage at the Pro-Pol site. These results suggest that nepoviruses share common mechanisms to regulate the processing of the RNA1 and RNA2-encoded polyproteins through the accumulation of proteinase precursors containing the VPg domain. However, these results also suggest that there are many variations on this theme, i.e. the effect of the VPg domain on the proteinase activity varies from one nepovirus to another. Interestingly, in potyviruses, although both the VPg-Pro (NIa) and the Pro (NIa-Pro) accumulate in plants, no differential activity was observed between them (Parks et al., 1992). Therefore, modulation of the proteinase activity by the VPg domain may be a characteristic feature of nepoviruses.

Acknowledgements

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