Inhibitory effects of human cystatin C on plum pox potyvirus proteases

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Abstract

The effect of different protease inhibitors on the proteolytic processing of the plum pox potyvirus (PPV) polyprotein has been analyzed. Human cystatin C, an inhibitor of cysteine proteases, interfered with the autoprocessing of the viral papain-like cysteine protease HCPro. Unexpectedly, it also had an inhibitory effect on the autocatalytic cleavage of the Nla protease which, although it has a Cys residue in its active center, has been described as structurally related to serine proteases. Other protease inhibitors tested had no effect on any of the cleavage events analyzed.

Proteolytic processing of polyproteins constitutes a largely employed mechanism in gene expression of plant and animal RNA viruses. In particular, plant potyviruses express their genome through a unique polyprotein which is processed by three virus-encoded proteases (Fig. 1; for a review see [26]). Proteinases P1 [24, 32] and HCPro [5] cleave at their respective carboxy ends, while protease Nla cleaves at seven sites characterized by conserved heptapeptides that differ in their susceptibility to be processed inter- or intramolecularly (trans or cis), as well as in the reaction profiles [7–10, 12–14, 17, 20]. Based on sequence analysis and site-directed mutagenesis, P1 has been proposed to be a serine protease extensively divergent from cellular proteases of this type [32], while HCPro was identified as a papain-like cysteine protease [19, 25]. On the other hand, Nla belongs to a group of viral proteases related to chymotrypsin-like cellular serine proteases that

![Fig. 1. Proteolytic processing of the potyviral protein. Cleavage events are represented by arrowed marks. The protein products identified in potyviral infections and those whose existence has been postulated relying on the characterization of cleavage sites are indicated in the lower part of the map. Since the Nla protein is a major product in infected cells, both the complete protein and its two domains, VPg and protease (Pro), which can be separated by a proteolytic cleavage (dashed line), are indicated in the map.](image-url)
Fig. 2. Effect of human cystatin C on PPV HCPro and Nla proteolytic activities. A and B. Fluorography of the translation products synthesized in a rabbit reticulocyte lysate system (Promega, used according to supplier's instructions) in the presence of 35S-methionine. Reactions were performed in the absence (lanes a) or in the presence (lanes b and c) of human cystatin C at the concentrations (µg/ml) indicated on the top of the lanes. The templates were synthetic RNAs obtained by in vitro transcription (as previously described [13]) of plasmids pGG5S6N, (linearized with Pvu II and Dra III, panel A) and pGGNla (with Pvu II and Hind III, panel B). These plasmids contain PPV cDNA sequences cloned in the pGEM 3 vector (Promega) under the control of a truncated T7 promoter [28]. The templates were used in the in vitro transcription system of a synthetic transcript encoding the complete P1 and HCPro proteins and 153 amino acids of the P3 protein (the full-length translation product has a molecular mass of 106.4 kDa) was used to study the autocatalytic processing of HCPro at its carboxy terminus. Only a major band, with an electrophoretic mobility similar to that expected for the P1-HCPro protein (87.6 kDa), was detected (Fig. 2A, lane a), suggesting that efficient processing at the HCPro C-end took place.

In vitro translation in a RR system of a synthetic transcript encoding the complete P1 and HCPro proteins and 153 amino acids of the P3 protein (the full-length translation product has a molecular mass of 106.4 kDa) was used to study the autocatalytic processing of HCPro at its carboxy terminus. Only a major band, with an electrophoretic mobility similar to that expected for the P1-HCPro protein (87.6 kDa), was detected (Fig. 2A, lane a), suggesting that efficient processing at the HCPro C-end took place. As previously reported for other potyviruses [24, 32], cleavage between P1 and HCPro did not occur in the RR

employed in this experiment. Human cystatin C purified according to Abrahamson et al. [1] was a generous gift from Dr. A. Gruub and Dr. M. Abrahamson.
system. The same processing pattern was obtained when the \textit{in vitro} translation was performed in the presence of 30 \mu g/ml of human cystatin C (Fig. 2A, lane b). However, in the presence of the inhibitor at 300 \mu g/ml, a protein of the size of the intact translation product was detected and the intensity of the band with the mobility corresponding to the P1-HCPro protein was much lower, indicating that proteolytic processing was inhibited. Densitometric analysis, taking into account the number of methionine residues in each protein, showed that about 50\% of the precursor was not processed. Leupeptin and aprotinin (Boehringer Mannheim) at 400 \mu g/ml had no appreciable effect on the processing by the HCPro protease (not shown).

The Nla protein has been shown to perform cis and trans proteolytic cleavages [4, 6, 12, 15]. We have studied the effect of protease inhibitors in the autocatalytic processing at the Nla-Nlb junction. \textit{In vitro} translation in a RR system of a synthetic RNA coding for Nla and 333 amino acids of the Nlb protein resulted in the appearance of two major polypeptides with the size corresponding to the products of processing of the encoded polyprotein at the Nla-Nlb junction (Fig. 2B, lane a). In the presence of human cystatin C at 300 \mu g/ml, a protein with the mobility expected for the intact translation product was detected (Fig. 2B, lane c), indicating that some inhibition of the cleavage was occurring. Densitometric analysis showed that about 13\% of the protein was not processed in the presence of the inhibitor. No alteration on the efficiency of translation in the RR system, at any of the concentrations of cystatin employed, was observed. Cystatin at a lower dose (30 \mu g/ml, Fig. 2B, lane b), or aprotinin or leupeptin at 400 \mu g/ml (not shown), did not affect the processing. No inhibitory effect of cystatin, leupeptin or aprotinin on the \textit{trans} cleavage at the Nlb-CP site, assayed by incubation of \textit{in vitro} translation products with extracts of \textit{Escherichia coli} expressing the Nla protease [13], could be detected (not shown).

The lack of significant inhibitory effects of aprotinin (inhibitor of serine proteases) and leupeptin (inhibitor of serine and cysteine proteases) on the \textit{trans} cleavage by the potyviral Nla protease at the Nlb-CP junction has been previously explained on the basis of the particular specificity of the Nla recognition sites [11]. With the results presented in this paper, this reasoning can now be extended to the \textit{cis} processing activity of the Nla protease and of the cysteine protease HCPro. Interestingly, human cystatin C, a known inhibitor of papain-like thiol proteases, efficiently interfered with the autoprocessing of the HCPro protease, indicating a close relationship between this protein and the cellular cysteine proteases. The case of the Nla protease is more complex. Based on sequence analysis it has been described that, although it contains in the active center a cysteine residue, this protease is much more related to serine proteases than to the cysteine ones [2, 18]. Since the mechanism of action of cystatins involves interactions with particular structural features of thiol proteases but does not produce a non-specific blockage of the active cysteine [30], it seemed unlikely that human cystatin C has an effect on Nla activities. Surprisingly, the experiments reported here indicate that the autocatalytic processing of the PPV Nla protease was little, but clearly, interfered by this inhibitor. This result could suggest for the Nla-type proteases an evolutionary position more equidistant between serine and cysteine protease than that currently accepted. However, due to the low level of inhibition and the high dose of inhibitor required to produce the effect, this conclusion should be considered only as tentative. On the other hand, preliminary results using a wheat germ \textit{in vitro} translation system seemed to indicate that P1 protease autoprocessing was not affected by either of the protease inhibitors employed in the experiments described in this paper (not shown), in agreement with its previous characterization as an atypical serine protease [24, 31, 32].

The inhibitory effects of human cystatin C on two highly specific proteases of PPV open the possibility to use it in strategies designed to interfere with potyvirus infections. Human cystatin C and rabbit cystatin have been shown to drastically reduce the replication of herpes simplex virus [3] and picornaviruses [21, 22]. Although
the proteolytic processing of potyvirus polyprotein likely consists of a highly regulated series of events and could be disturbed without completely abolishing the protease activity, the high dosis of cystatin required to observe an inhibitory effect suggests that it probably could not be actually used as an antipotyviral agent. However, it is probable that further studies on structure-function relationships and site-directed mutagenesis could allow the design of highly specific inhibitors of the potyviral proteases with low collateral effects on the cellular physiology.

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