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Virus Research 110 (2005) 91–97
Virus Research
www.elsevier.com/locate/virusres

Characterization of the norovirus 3C-like protease

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Received 17 September 2004; received in revised form 8 February 2005; accepted 8 February 2005
Available online 8 March 2005

Abstract

The recombinant 3C-like protease of Chiba virus, a Norovirus, expressed in *Escherichia coli* cells was purified and characterized as to effects of pH, temperature, salt contents, and SH reagents on its proteolytic activity. The optimal pH and temperature of the 3C-like protease for the proteolytic activity were 8.6 and 37 °C, respectively. Increased concentration (∼100 mM) of monovalent cations such as Na^+ and K^+ was inhibitory to the activity. Hg^{2+} and Zn^{2+} remarkably inhibited the protease activity, while Mg^{2+} and Ca^{2+} had no virtual effect. Several sulfhydryl reagents such as *p*-chloromercuribenzoic acid, methyl methanethiosulfonate, *N*-ethylmaleimide and *N*-phenylmaleimide also blocked the activity, confirming the previous result that cysteine residue(s) were responsible for the proteolysis.

Keywords: 3C-like protease; Chiba virus; Norovirus; SH reagents; Proteolytic activity

1. Introduction

Viral proteases play a central role in the maturation of functional viral proteins, and hence in its genome replication and the formation of virus particles (Dougherty and Semler, 1993). The 3C and 3C-like proteases belonging to the chymotrypsin-like protease superfamily are found in several animal, insect, and plant virus families such as *Picornaviridae*, *Comoviridae*, *Caliciviridae*, *Coronaviridaee*, and *Arteriviridae* (Dougherty and Semler, 1993; Gorbalenya et al., 1989). Their three-dimensional structures have been determined by X-ray crystallography in poliovirus (Mosimann et al., 1997), rhinovirus (Matthews et al., 1994), hepatitis A virus (Bergmann et al., 1997), and coronavirus (Anand et al., 2002, 2003; Yang et al., 2003), tobacco etch virus (Phan et al., 2002) and equine arteritis virus (Barrette-Ng et al., 2002). These studies allowed us to identify the active-site amino acid residues with a combination of site-directed mutagenesis studies (Boniotti et al., 1994; Cheah et al., 1990; Gosert et al., 1997; Hammerle et al., 1991). Several 3C and 3C-like proteases were purified and their biochemical properties were well characterized (Baum et al., 1991; Chisholm et al., 2001; Davis et al., 1997; Ziebuhr et al., 1997). They are characterized by their proteolytic activities being inhibited by both cysteine and serine protease inhibitors. As a result of these studies, therapeutic drugs, especially for rhinovirus, are being developed (Hammerle et al., 1991; McKinlay, 2001; Turner, 2001).

Norovirus (formerly Norwalk-like virus) is a genus of the family *Caliciviridae* and is a major causative agent of non-bacterial acute gastroenteritis in humans (Clarke et al., 1998; Estes et al., 1997). Norovirus cannot be propagated in cell cultures, and animal models have not been developed, which hampers the progress of biochemical and molecular biological studies of Norovirus. We cloned and sequenced the whole genome of a Chiba strain of Norovirus (Chiba virus), and its 3C-like protease has been heterologously expressed in *Escherichia coli* in an active form (Someya et al., 2000). Furthermore, we identified the active-site amino acid residues and concluded that Norovirus 3C-like protease had a catalytic dyad consisting of His30 and Cys139 (Someya et al., 2002). This is a unique feature since most of the 3C and 3C-like proteases have a catalytic triad consisting of His, Asp/Glu, and Cys/Ser, similar to chymotrypsin (Dougherty and Semler, 1993; Gorbalenya et al., 1989). More recently, it was

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doi:10.1016/j.virusres.2005.02.002

shown that the active site of Coronavirus 3C-like protease was also composed of two residues, His and Cys (Anand et al., 2002).

To obtain information regarding the biochemical properties of norovirus 3C-like protease, we purified the bacterially expressed recombinant 3C-like protease from Chiba virus and characterized as to effects of pH, temperature, salt contents, and SH reagents on its activity.

2. Materials and methods

2.1. Construction of plasmids

The DNA fragment encoding all residues (Ala1 to Glu181) of the Chiba virus 3C-like protease was amplified by PCR, in that *Nde*I and *Aor*51HI restriction sites were introduced at the 5′ and 3′ ends, respectively. The *Nde*I–*Aor*51HI fragment was placed downstream of the T7 promoter with a linker encoding a sequence containing six consecutive His residues. The resultant plasmid, pT7ProHis, encodes a 3C-like protease with a Met residue in the N-terminal and with a SAGHHHHHHG sequence in the C-terminal. The *Nde*I–*Sph*I fragment from pUCHis3CD (Someya et al., 2000) encoding the 3CD region was placed downstream of the T7 promoter to construct pT7His3CD. Next, the C139A mutation of the 3C-like protease was introduced and designated pT7His3CD-C139A. The DNA fragment encoding glutathione *S*-transferase (GST) was amplified by PCR, in that *Apa*I and *Sph*I restriction sites were introduced at the 5′ and 3′ ends, respectively. The *Apa*I–*Sph*I fragment encoding GST was ligated to the *Apa*I–*Sph*I fragment of pT7His3CD-C139A. The resultant plasmid was designated pT7His3Cd-GST. This plasmid encodes the N-terminal His-tag sequence (MGGHHHHHHGASA), followed by the whole sequence of the C139A mutant 3C-like protease and Gly1 to Pro19 of 3D RNA-dependent RNA polymerase, and then followed by GST.

2.2. Purification of proteins

E. coli BL21-CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA) were transformed with pT7ProHis or pT7His3Cd-GST. Cells were grown on medium A (McMurry et al., 1980) supplemented with 0.2% glucose and 0.1% casamino acids at 37 °C. When the absorbance at 530 nm reached 0.5, IPTG was added to a final concentration of 1 mM for induction of gene expression. After 2 h, cells were harvested, then washed and resuspended with 20 mM Tris–HCl (pH 8.0) buffer containing 0.1 M NaCl. Cells were disrupted by sonication. After the removal of cell debris, the supernatant was subjected to ultracentrifugation (150,000 × *g*, 1 h). The resultant supernatant was mixed with TALON Metal Affinity Resin (CLONTECH, Palo Alto, CA) equilibrated with 20 mM Tris–HCl (pH 8.0) buffer containing 0.1 M NaCl. The resin was washed with 20 mM Tris–HCl (pH 8.0) buffer containing 0.1 M NaCl and 0.1 M imidazole for removal of non-specifically bound proteins. The proteins of interest were eluted with 20 mM Tris–HCl (pH 8.0) buffer containing 0.1 M NaCl and 0.5 M imidazole and concentrated using Ultrafree-15 unit with Biomax-10 membrane (MILLIPORE, Bedford, MA). Proteins were determined by the method of Bradford (Bradford, 1976).

2.3. Proteolytic reaction

Usually, 5 μM enzyme and 5 μM substrate were reacted in 20 μl of the mixture containing 50 mM bis–tris–propane–HCl buffer (pH 8.62) for 16 h. Details of reaction mixtures were described in the respective sections. The reaction was stopped by the addition of an equal volume of 2× sample buffer, and the proteins were then analyzed by SDS-polyacrylamide (13%) gel electrophoresis. Gels were stained with Coomassie Brilliant Blue R-250. In order to estimate the efficiency of the proteolytic cleavage, density of the respective stained bands was scanned and calculated using NIH Image for Macintosh version 1.62.

3. Results and discussion

3.1. Choice of the substrate protein

The viral non-structural proteins and the proteolytic cleavage sites within the ORF1 polyprotein are depicted in Fig. 1A. In order to purify the Chiba virus 3C-like protease, we constructed pT7ProHis with the gene fragment encoding the protease with His-tag in its C-terminus (Fig. 1B). The ProHis protein (calculated molecular mass of 20.6 kDa) expressed from pT7ProHis was used as an enzyme in this study. In order to observe proteolysis at the cleavage site between the 3C and 3D, we at first used the His-3CD-C139A mutant protein expressed from pT7His3CD-C139A as a substrate, which was the N-terminal His-tagged 3CD fragment containing the Ala mutation of active-site Cys139 of the 3C-like protease (Fig. 1B). However, this protein tended to aggregate at higher pH after prolonged incubation. Other parts from the native Chiba virus ORF1 polyprotein could not be effectively produced in *E. coli* cells. Therefore, the His3Cd-GST protein (calculated molecular mass of 48.0 kDa) was used as a substrate, which retains the cleavage site between the 3C and 3D. The region covering the entire 3C and the N-terminal 19 amino acids of 3D was placed under the control of T7 promoter with the N-terminal His-tag and the C-terminal glutathione *S*-transferase (GST), the resultant plasmid being designated pT7His3Cd-GST (Fig. 1B). Although this protein itself has the 3C-like protease moiety, it never undergoes autocatalytic cleavage because the C139A mutation is introduced. If His3Cd-GST is cleaved at the 3C/3D junction (LE/GG) by the active ProHis, the His3C-C139A moiety of 20.7 kDa and the 3D′-GST moiety of 27.3 kDa are produced. Enzymatic reaction was initiated