Virology 438 (2013) 20–27

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

# RNA binding by human Norovirus 3C-like proteases inhibits protease activity

Prasanth Viswanathan, Jared May, Sunghae Uhm, Changsuek Yon, Brent Korba *

*Georgetown University Medical Center, Department of Microbiology and Immunology, 3900 Reservoir Rd., N.W., Med-Dent Building, RM SW319, Washington, DC 20057, United States*

ARTICLE INFO

*Article history:*
Received 15 October 2012
Returned to author for revisions
20 November 2012
Accepted 11 January 2013
Available online 9 February 2013

*Keywords:*
Norovirus
Protease
RNA binding

ABSTRACT

A highly active, fluorescence-based, *in vitro* assay for human Norovirus protease from genogroup I and II viruses was optimized utilizing as little as 0.25 μM enzyme, pH 7.6, and substrate:enzyme of 50–100. Activity in Tris–HCl or sodium phosphate buffers was 2-fold less than HEPES, and 2-fold lower for buffer concentrations over 10 mM. Protease activity at pH 7.6 was 73% (GI) or 63% (GII) of activity at the optimal pH 9.0. Sodium inhibited activity 2–3 fold, while potassium, calcium, magnesium, and manganese inhibited 5–10 fold. Differences in efficiency due to pH, buffer, and cations were due to changes in $k_{cat}$ and not $K_m$. Norovirus protease bound short RNAs representing the 3′ or 5′ ends of the virus, inhibiting protease activity ($IC_{50}$ 3–5 μM) in a non-competitive manner. Previous reports indicated participation of the protease in the Norovirus replicase complex. The current studies provide initial support for a defined role for the viral protease in Norovirus replication.



## Introduction

Noroviruses are a group of related non-enveloped, single-stranded, positive sense RNA viruses that cause acute gastroenteritis in humans. Noroviruses are the most common cause of epidemic gastroenteritis, responsible for at least 50% of all gastroenteritis outbreaks worldwide, while 1 in 15 (21 million) residents in the United States acquire these infections annually (Hall et al., 2011; Scallan et al., 2011). An estimated 9.4 million episodes of foodborne illness occur in the United States every year and 5.5 million (58%) are caused by Noroviruses (Hall et al., 2011; Scallan et al., 2011). Noroviruses belong to the genus *Norovirus*, of the family Caliciviridae (Kapikian et al., 1997; Zheng et al., 2006). At least five genogroups (GI–GV) of Noroviruses have been recognized based on the amino acid identity in the capsid protein, VP1, with the human Noroviruses belonging to groups GI, GII, and GIV (Kapikian et al., 1997; Zheng et al., 2006). GII viruses have emerged as the most prevalent cause of disease (Hall et al., 2011; Scallan et al., 2011).

The prototype human Calicivirus, Norwalk Virus, has a 7.7 kb positive sense single-stranded RNA genome that encodes three open reading frames (ORFs) (Kapikian et al., 1997). ORF 1 encodes a 200 KDa polyprotein, which is cleaved by the 3C-like cysteine protease of the virus into six non-structural proteins necessary for viral replication, one of which is the viral protease. The absolute requirement of the protease of Noroviruses (NoV *pro*), makes it an attractive target for antiviral intervention (Blakeney et al., 2003; Hardy et al., 2002; Tiew et al., 2011). A crystal structure of the Norwalk virus 3C protease has been solved and mutational analysis has identified the amino acids critical for activity (Nakamura et al., 2005; Someya et al., 2008; Someya and Takeda, 2011; Zeitler et al., 2006). A peptide mimic inhibitor of the Southampton Norovirus 3C protease has been modeled into a crystal structure that provides further insight into functional residues (Zeitler et al., 2006).

We have developed a highly active fluorescence-based *in vitro* assay to quantitatively measure the activity of NoV *pro* for both the GI and GII viruses that is notably more robust than most previously reported assays (Belliot et al., 2003; Blakeney et al., 2003; Chang et al., 2012; Hardy et al., 2002; Hussey et al., 2011; Scheffler et al., 2007; Tiew et al., 2011; Someya et al., 2008: Someya and Takeda, 2011; Zeitler et al., 2006). This assay operates in a 96-well format with low enzyme concentrations, produces a high signal-to-background ratio, is highly reproducible, and was used to characterize buffer, ion, and pH requirements of the protease, which have not been systematically examined in previous reports. To provide a basis for the mechanisms involved in the observed effect on activity, we have determined that these components affect catalytic activity ($k_{cat}$), but not binding affinity for the peptide substrate ($K_m$).

Our examination of the published crystal structure of NoV *pro* revealed a potential ATP binding motif. Since NoV *pro* is hypothesized to directly participate in the viral replicase (Belliot et al., 2005, 2008; Cancio-Lonches et al., 2011; Machín et al., 2009; Wei et al., 2001), we investigated the interaction of RNA oligomers representing the 3′ and 5′ ends of the viral genome and NTPs with the viral protease. We provide the first evidence for direct RNA

* Corresponding author. Fax: +1 202 687 1800.
*E-mail address:* korbabe@georgetown.edu (B. Korba).

0042-6822/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.virol.2013.01.006

binding by NoV *pro* and demonstrate that RNA binding efficiently inhibits protease activity. These observations provide initial evidence for a defined role of NoV *pro* in the viral replicase, possibly similar to that observed for Rhinovirus and Poliovirus (Andino et al., 1993; Hammerle et al., 1992; Leong et al., 1993; Matthews et al., 1994).

## Results

### *Cations and buffer composition reduce NVpro activity*

Nearly all previously published human Norovirus protease (NoV *pro*) assays have utilized 50–100 mM sodium phosphate buffer, pH 8.0 or greater, NaCl at 100–150 mM, enzyme concentrations of 2 μM or greater, and relatively low substrate:enzyme ratios (less than 10) (Belliot et al., 2003; Blakeney et al., 2003; Chang et al., 2012; Hardy et al., 2002; Hussey et al., 2011; Scheffler et al., 2007; Tiew et al., 2011; Someya et al., 2008: Someya and Takeda, 2011; Zeitler et al., 2006). However, there is little information on the relative effect of alternative assay conditions, or a systematic examination of reaction conditions and components. The standard buffer composition for the NoV *pro* activity assay in the current study (10 mM HEPES, pH 7.6, 0.1% CHAPS, 5 mM DTT, 30% glycerol) was based on empirical observations that examined the effect of pH, detergent, buffering agent, and commonly utilized cations.

Activity of NoV *pro* from both genogroup *I* (GI) and II (GII) viruses was examined and was found to be essentially identical overall. Protease activity was maximal at pH 8.6–9.0, but strong protease activity at more physiologically relevant levels was retained (Fig. 1A). At pH 7.6, the GI *pro* retained 73% and the GII *pro* retained 63% of the activity at the optimal pH (Fig. 1A). Protease activity in HEPES was at least 2-fold higher than in Tris–HCl or $NaPO_4$ across a range of pH values (Fig. 1B,C). Protease activity at buffer concentrations higher than 10 mM was reduced for all three buffering agents examined at pH 7.6 (Fig. 1C).

For the GI *pro*, NaCl inhibited activity 2-fold at approximately 90 mM, but higher concentrations up 300 mM had little additional effect (Fig. 2A). KCl, $MgCl_2$, $MgSO_4$, $CaCl_2$, and $MnCl_2$ were more inhibitory, inducing a 2-fold reductions at as little as 5–10 mM in some instances, and up to 10-fold at higher concentrations (Fig. 2A,B). Relative to the GI protease, the GII enzyme appeared to be slightly more sensitive to NaCl and $MgCl_2$ and slightly less sensitive to $MgSO_4$ and $CaCl_2$ (Fig. 2A,B). Data for zinc is not included as addition at any concentration induced precipitation.

While the addition of CHAPS and DTT only modestly enhanced GI *pro* activity (with a greater apparent effect on GII *pro* at lower concentrations) (Fig. 2C,D), DTT and CHAPS were included in the final reaction buffer to potentially aid solubility and stability of the enzyme. EDTA at concentrations up to 0.5 μM, and DMSO at concentrations of up to 5%, did not affect protease activity (data not shown).

In the standard assay buffer, NoV *pro* concentrations as low as 0.25 μM consistently produced signals at least 2-fold higher than background (no enzyme) control reactions (Fig. 3, top panel). Enzyme concentrations of 0.5 μM and 1.0 μM were much superior to lower concentrations. Maximal efficiency of the reaction was observed at a 100-fold excess of substrate relative to NoV *pro* (Fig. 3, bottom panel).

### *NVpro binds RNA which inhibits protease activity*

Previous studies have provided evidence that Norovirus and other Calicivirus proteases participate in the viral replicase complex either as the mature cleaved protein (*pro*) or the un-cleaved

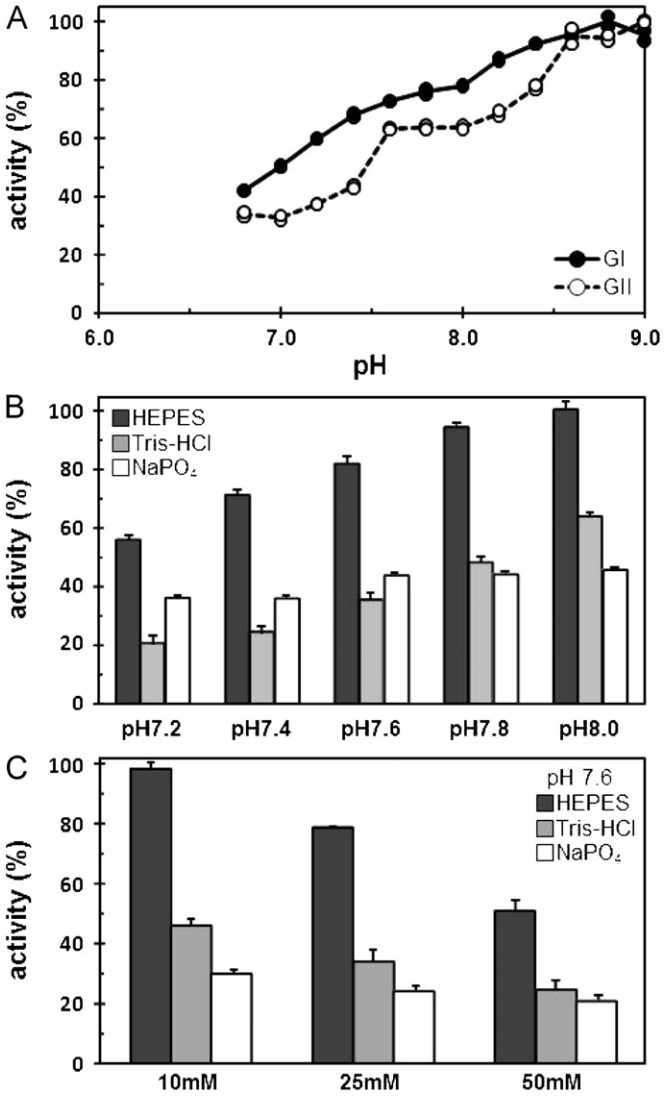


**Fig. 1.** Effect of pH and buffering agent on NoV *pro* activity. NoV *pro* concentration was 0.5 μM and substrate was held at 100-fold excess for all conditions. Values were determined at 60 min of reaction, and values for duplicate reactions are presented. Panel A: Effect of pH. Data are presented as a percentage of the maximum value. Assays were conducted in 50 mM Tris–HCl. Panel B: Effect of buffering agent on GI *pro*. Buffering agents were used at 50 mM. Data are presented (in duplicate at each point) as a percentage of the highest assay value observed (HEPES, pH 8.0, set at 100). Bars denote standard deviations. Panel C: Effect of buffer concentration. Assays were conducted at pH 7.6. Data are presented as a percentage of the highest assay value observed (10 mM HEPES, set at 100%). Bars denote standard deviations. Maximum net RLU (after background subtraction) for each panel were: A, 1642(GI), 2015(GII); B, 1693(GI); C, 1439(GI).

precursor protein (*propol*) (Belliot et al., 2005, 2008; Cancio-Lonches et al., 2011; Machín et al., 2009; Wei et al., 2001) but no distinct role for protease in this complex has been demonstrated. Our examination of the amino acid sequence and three dimensional structure of NoV *pro* (SitePredict™, http://sitepredict.org) indicated the presence of a potential ATP binding pocket defined by the following amino acids: M107, I109, Q110, R112, V114, S118, L121, G133, T134, I135, P136, G137, D138, C139, H157, A158, A159, A160, T161, K162, S163, G164, N165, T166, V167, V168. The effects of NTPs on protease activity were subsequently examined. All four NTPs appeared to weakly inhibit GI *pro* activity ($IC_{50}$ 1.2–2.9 mM), but did not inhibit activity substantially further at increasing concentrations up to 10 mM