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Short communication

Expression and biochemical characterization of nsP2 cysteine protease of Chikungunya virus

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Abstract

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that causes epidemic fever, rash and polyarthralgia in Africa and Asia. Although it is known since the 1950s, new epidemiological and clinical features reported during the recent outbreak in the Indian Ocean can be regarded as the emergence of a new disease. Numerous severe forms of the infection have been described that put emphasis on the lack of efficient antiviral therapy. Among the virus-encoded enzymes, nsP2 constitutes an attractive target for the development of antiviral drugs. It is a multifunctional protein of approximately 90 kDa with a helicase motif in the N-terminal portion of the protein while the papain-like protease activity resides in the C-terminal portion. The nsP2 proteinase is an essential enzyme whose proteolytic activity is critical for virus replication.

In this work, a recombinant CHIKV nsP2pro and a C-terminally truncated variant were expressed in *Escherichia coli* and purified by metal–chelate chromatography. The enzymatic properties of the proteinase were then determined using specific synthetic fluorogenic substrates. This study constitutes the first characterization of a recombinant CHIKV nsP2 cysteine protease, which may be useful for future drug screening.

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Chikungunya virus (genus *Alphavirus*, family *Togaviridae*) is transmitted by *Aedes* mosquitoes (Porterfield, 1980; Strauss and Strauss, 1986) and is typically responsible in human of an acute illness, which includes fever, maculopapular rash, and incapacitating arthralgia (Robinson, 1955; Johnston and Peters, 1996; Jupp and Cornel, 1988). Isolated for the first time during a Tanzanian outbreak in 1952 (Ross, 1956), CHIKV is geographically distributed in Africa, India, and Southeast Asia (Lam et al., 2001; Pastorino et al., 2004; Saluzzo et al., 1983). In Africa, until 2000, it was described as endemic through a sylvatic cycle involving wild primates, humans and mosquitoes of the genus *Aedes* (Powers et al., 2000). However, during the

last 6 years, large urban outbreaks have been also reported in central Africa (Pastorino et al., 2004). Due to recent extension around the Indian Ocean, including Comoros, Mauritius, Reunion island, Madagascar and India (Bessaud et al., 2006b; Charrel et al., 2007; Schuffenecker et al., 2006; Ravi, 2006), and now in Italia, CHIKV is regarded as a potential worldwide public health problem, with no preventive or therapeutic means available.

Both Sindbis virus (SINV) and Semliki Forest virus (SFV) have been studied extensively at the molecular level. They have served as models for determining alphavirus architecture and replication strategy. The genome of alphaviruses is a single RNA molecule of positive polarity, about 11.5 kb in length, encoding four non-structural proteins (nsP1–4) that are the essential components of the viral replicase and transcriptase. Functional proteins are first expressed as a polyprotein that is then cleaved by the viral proteases.

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The nsP2 protein is multifunctional (Hardy and Strauss, 1989; Merits et al., 2001; Strauss et al., 1992; Vasiljeva et al., 2001). Nucleoside triphosphatase, helicase, and RNA-dependent 5'-triphosphatase activities are located in the N-terminus of the protein while the proteolytic domain has been mapped to its C-terminal part. It forms a papain-like thiol protease with a catalytic dyad involving two conserved residues C and H. The nsP2 protease is responsible for cleavages in the non-structural polyprotein (Kim et al., 2004; Vasiljeva et al., 2003) that are crucial for the viral replication cycle. Therefore, this protease constitutes an attractive target for the development of antiviral compounds.

Previous functional mapping of SFV nsP2 (Vasiljeva et al., 2001) had defined a soluble nsP2 region with protease activity and called Pro39 (SFV residues M⁴⁵⁹–C⁷⁹⁹). The CHIKV nsP2pro sequence was determined by alignment of CHIKV (GenBank accession number AF369024), SINV (AF429428) and SFV (NC003215) amino acid sequences (residues E⁴¹⁷–C⁷⁹⁹) using the Clustal X 1.81 software (Thompson et al., 1997). The overall nsP2pro sequence conservation was about 38% at the nucleotidic level and 63% at the amino-acidic level when CHIKV sequence was compared to SFV. The catalytic residues C⁴⁷⁸ and H⁵⁴⁸ were found invariant in all the three nsP2pro sequences, as well as the W⁵⁴⁹, which has been shown to be necessary for proteolytic activity (Golubtsov et al., 2006; Strauss et al., 1992).

Based on this sequence analysis, we constructed two specific plasmids using the pET-DEST42 Vector, following specifications of Gateway Cloning Technology (Invitrogen, Cergy pontoise, France). The primers CHIK-D1 (genome position 2948–2965): 5'-AAAAAGCAGGCTCTGAAGGAGATAGAACCATGTGGAAAGACACTCTCCGGT and CHIK-C2 (genome position 4030–4048): 5'-AGAAAGCTGGGTAACATCCTGCTCGGGTGGC were used for the first construction (nsP2pro) expressing the CHIKV nsP2pro from residue W⁴²² through residue C⁷⁹⁹. The primers CHIK-D1 and CHIK-C1 (genome position 3563–3581): 5'-AGAAAGCTGGTAGACCAGGAGCACGTGGTG were used for the second construct (nsP2proDel) (from residue W⁴²² through residue V⁶³⁹).

CHIKV nsP2pro and nsP2proDel were expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene) with 1 mM IPTG for 6 h at 22 °C in TB media (Fig. 1). The two recombinant proteins were partially soluble. Different conditions were tested (addition of denaturing agents, detergents or glycylglycine (Ghosh et al., 2004)) but failed to improve the protein solubility.

Purification was performed using metal–chelate chromatography (Ni-NTA, Qiagen, Courtabœuf, France) loaded onto a XK16/20 column (Amersham Biosciences, Orsay, France) connected to an ÄKTA_{FPLC} system (Amersham Biosciences). After purification, the Coomassie-stained SDS-PAGE revealed additional bands accompanying the recombinant proteins. The use of Co²⁺ matrix and the increase of the wash steps stringency, as well as size exclusion (Superdex 200 10/300 column, Amersham) and ion exchange (mono Q 5/50 column, Amersham) chromatographies did not improve the purity rate. The main contaminating proteins were identified by MALDI-TOF-MS analysis as the chaperone protein htpG of *E. coli* (molecular weight 71,378 Da, number Swiss-Prot/TrEMBL: HTPG_ECO57), already described when recombinant proteins are overproduced in *E. coli* (Rikkonen et al., 1992; Liberek et al., 1991; Sherman and Goldberg, 1992), and as the elongation factor EFCA of *E. coli* (molecular weight 42,422 Da); these proteins do not display any protease activity. Washing the Ni-NTA purified fractions with MgATP (Rial and Ceccarelli, 2002) for the purpose of eliminating the chaperone protein htpG resulted in a concomitant loss of a substantial proportion of the recombinant protein (data not shown).

The identity of nsP2pro and nsP2proDel were verified by immunoblotting using anti-His monoclonal antibodies (Fig. 1). After the purification steps, nsP2pro and nsP2proDel protein concentration was determined to be ~3 μM with a purity close to 70% using a Lumi-Imager imaging system (Roche Applied Sciences, Meylan, France). Purified protein samples were then concentrated using Vivaspin concentrators (Vivascience, Hannover, Germany).

To characterize the enzymatic activity of the purified protease nsP2pro and nsP2proDel, we investigated their ability to cleave peptidyl-7-amino-4-methylcoumarine-labelled substrates; this kind of substrate had been previously used to study the activ-

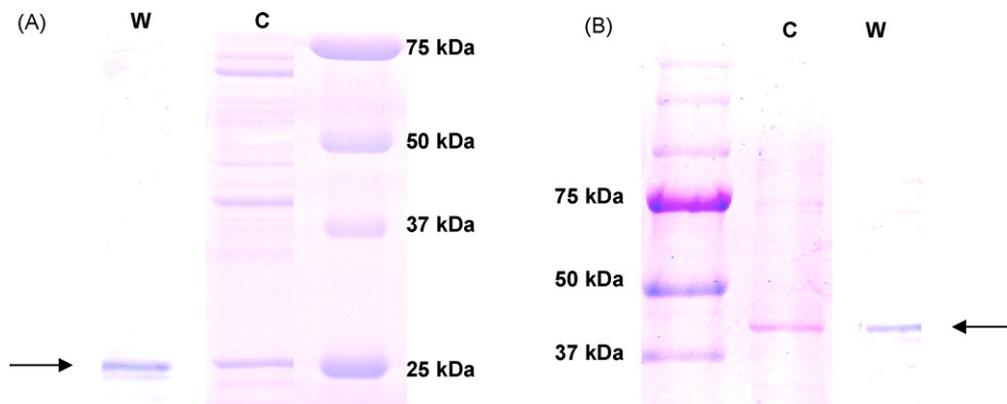


Fig. 1. Analysis by Coomassie blue staining (C) and Western blotting (W) of the purified nsP2proDel (A) and nsP2pro (B) recombinant proteins. Arrows indicate the recombinant proteins after immobilized-metal affinity chromatography.

ity of a wide range of proteases, such as eukaryotic and viral trypsin-like proteases (Jeohn et al., 1999; Kato et al., 1998; Bessaud et al., 2006a; Yusof et al., 2000) and cysteine proteases (Choe et al., 2006; Melo et al., 2001; Gosalia et al., 2005). Such short substrates are useful to assay the catalytic activity of proteases and to map their specificity at non-prime sites. Residues in the P₄-P₃-P₂-P₁ positions (according to Schechter & Berger's nomenclature, 1967) are thought to be important for nsP2 cleavage as the enzyme can only recognize substrates with small residues in these positions and specifically a G in the P₂ site (Lulla et al., 2006). Taking into account these data, we designed two synthetic peptides, Boc-AGA-MCA and Boc-AGG-MCA (JPT Peptide Technologies, Berlin, Germany), which represent the natural non-prime part of the CHIKV nsP1/nsP2 and nsP3/nsP4 cleavage sites, respectively.

Both the two substrates were found to be cleaved by nsP2pro. In contrast, no activity on Boc-AGA-MCA and Boc-AGG-MCA substrates was detected using the nsP2proDel protease even at an enzymatic concentration up to 5 μ M. This result confirmed the functional importance of the nsP2 C-terminal region for its protease activity and the absence of protease contaminating activity in the recombinant protein preparation. Previous studies had shown that SFV nsP2 protease activity was inhibited by deletion in the 200 carboxyl-terminal domain (Vasiljeva et al., 2001). Recently, the Venezuelan equine encephalitis virus (VEEV) nsP2 protease structure has been resolved (Russo and Watowich, 2006) and provided evidence that the C-terminal moiety may contribute to substrate recognition. Thus, the C-terminal region of alphavirus nsP2pro could fill a role similar to the NS2B cofactor of the flavivirus-encoded NS3 protease (Aleshin et al., 2007).

The tri-peptide substrate Boc-AGG-MCA was used to determine the optimal conditions for enzymatic activity of nsP2pro. The pH dependence of proteolytic processing was studied at constant ionic strength and the optimal pH for enzyme catalysis was determined to be 9.5 (Fig. 2A). Enzymatic activity was higher than 80% between pH 8 and 10 and decreased rapidly out of this range. It was already the case for others purified viral recombinant proteases (Bessaud et al., 2006a; Li and Singh, 1999; Nall et al., 2004; Shiryayev et al., 2006). The existence of such an alkaline optimal pH could indicate that proteolysis is a regulated process *in vivo* at physiological pH of the cytoplasm.

The CHIKV protease activity was slightly inhibited by NaCl (Fig. 2B): the optimal activity values were obtained for NaCl concentration between 10 and 50 mM. Increasing salt concentration up to 250 mM resulted in a 50% decrease of the protease activity. This result can be explained by the importance of electrostatic interactions in cysteine protease/substrate binding.

Overproduced recombinant proteins had a tendency to aggregate and it had been shown that addition of glycerol or a neutral detergent could enhance the targeted activity by increasing accessibility of the enzyme site to substrate (Bessaud et al., 2005; Leung et al., 2001; Nall et al., 2004). However, CHIKV nsP2pro processing activity was significantly inhibited by glycerol addition: at glycerol concentration 10%, the protease activity was already decreased about 50% and dropped to 20% with 20% glycerol (Fig. 2C).

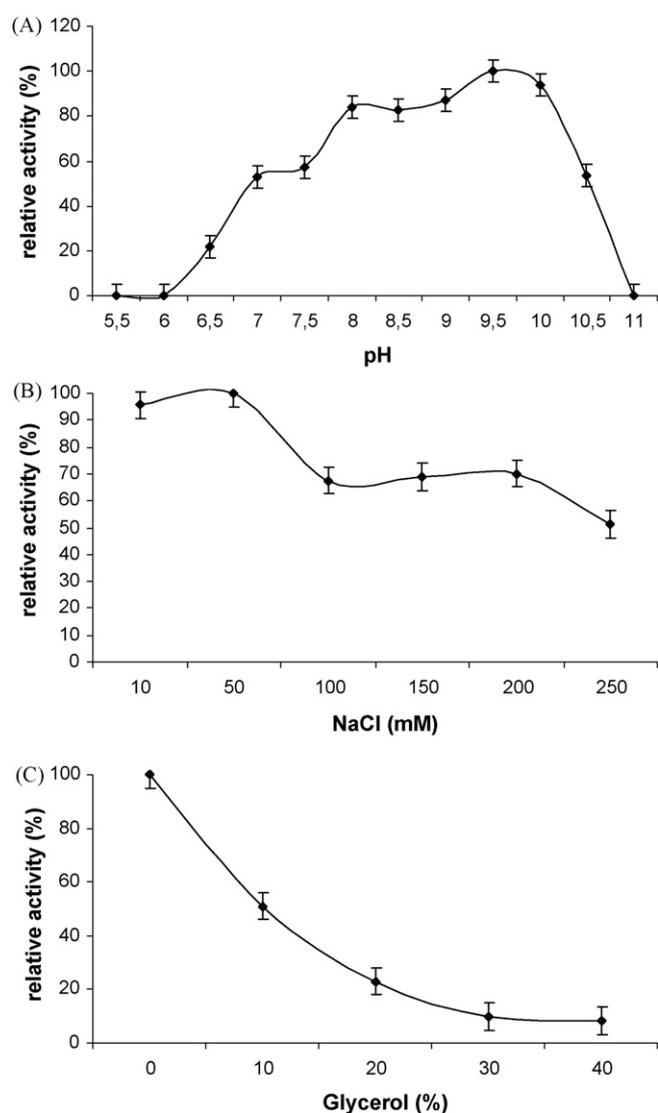


Fig. 2. Enzymatic characterization of CHIKV nsP2pro. Effects of pH, ionic strength and glycerol content on proteolytic processing by CHIKV nsP2pro protein. (A) Effect of pH; for each data point, relative activity was defined as the ratio $(100 \times V_i)/(V_i)_{\text{pH } 9.5}$. (B) Effect of ionic strength, observed in buffers containing Tris 50 mM pH 9.5 and varying concentration of NaCl; for each data point, relative activity was defined as the ratio $(100 \times V_i)/(V_i)_{10 \text{ mM}}$. (C) Effect of glycerol; for each data point, relative activity was defined as the ratio $(100 \times V_i)/(V_i)_{0\%}$.

Therefore, the optimal experimental conditions were determined to be Tris 50 mM pH 9.5, NaCl 10 mM.

Under these optimal conditions, the purified CHIKV nsP2pro protease showed a 4.5-fold reduced activity for substrate Boc-AGA-MCA compared to Boc-AGG-MCA (Table 1). For Boc-AGA-MCA substrate, the K_m and k_{cat} were, respectively 10 times and 2.5 times higher than the values obtained with the Boc-AGG-MCA substrate. The kinetic comparison showed that the CHIKV nsP2pro protease had a higher substrate affinity for the Boc-AGG-MCA substrate resulting in a more efficient cleavage. This result is consistent with the alphavirus replication cycle in which the speed of nsP1234 processing plays a crucial role in the control of RNA synthesis (Lemm and Rice, 1993; Lemm et al., 1994; Shirako and Strauss, 1994). In alphavirus-

Table 1
Kinetic parameters of the degradation of selected fluorogenic substrates by CHIKV nsP2 protease

Substrate	Junction	K_m (μM)	k_{cat} ($\times 10^{-6} \text{ s}^{-1}$)	k_{cat}/K_m ($\text{s}^{-1} \text{ M}^{-1}$)
Boc- AGA -MCA	nsP1/nsP2	2,422	932	0.38
Boc- AGG -MCA	nsP3/nsP4	232	374	1.61
Dabcyl- AGAGIIE -Edans	nsP1/nsP2	ND	ND	ND
Dabcyl- AGGYIFSE -Edans	nsP3/nsP4	61	140	2.3

Non-prime substrate sequences are indicated in bold and prime substrate sequences are shown in italics. Protease activity was assayed in Tris 50 mM pH 9.5, NaCl 10 mM using a substrate concentration ranging from 25 to 200 μM . Standard reaction contained the CHIKV nsP2pro protease at a concentration of 0.5 μM . The kinetic parameters for the degradation of Dabcyl-**AGAGIIE**-Edans have not been determined (ND) because of the too low activity of the protease towards this substrate.

infected cells, the non-structural polyprotein is cleaved at three sites in the specific order 3/4–1/2–2/3. The 3/4 site is rapidly cleaved during infection, and this cleavage is absolutely essential for virus replication and virus-specific RNA synthesis (Kim et al., 2004; Lemm et al., 1994). Similar data have been previously reported with nsP2 proteases of SFV and SINV (Lulla et al., 2006; Vasiljeva et al., 2001).

Our optimised assay was also performed using longer quenched fluorogenic hepta- and octa-peptides (Eurogentec S.A., Liège, Belgium) overlapping the natural cleavage sites 1/2 and 3/4 (Table 1), in order to identify the role of flanking sequences for proteolytic activity. The nsP2proDel protein was inactive on these substrates whereas enzymatic activity was detected using the nsP2pro recombinant protease. The highest activity was obtained using the fluorogenic peptide overlapping the nsP3/nsP4 natural cleavage site, confirming the previous result obtained with the MCA peptides. The CHIKV protease showed a higher affinity for this more authentic substrate than for the shorter Boc-AGG-MCA while the k_{cat}/K_m ratio was almost similar.

The hepta-peptide substrate corresponding to the natural nsP1/nsP2 cleavage site was very poorly cleaved; therefore, we were not able to determine the corresponding enzymatic constants for its degradation. This result could be the consequence of the poor substrate solubility due to a relatively high rate of hydrophobic residues (A, F, I, Y) in the hepta-peptide sequence (Pettrassi et al., 2005).

As an insight into the protease catalytic mechanism, the effect of different protease inhibitors on the enzymatic activity of CHIKV nsP2pro was also tested, using the cost-effective Boc-AGG-MCA peptide as substrate (Fig. 3). As expected, the enzyme was completely resistant to the inhibitors of serine proteases (AEBSF, aprotinin), aspartic proteases (pepstatin), metalloproteases (EDTA). It was also resistant to one cysteine protease inhibitor (leupeptin). However, cleavage of the Boc-AGG-MCA substrate was completely inhibited by 25 mM *N*-ethylmaleimide (NEM) and by 2 mM of divalent cations Zn^{2+} or Cu^{2+} . These results are consistent with the classification of nsP2 as a thiol proteinase of the papain superfamily and with data already described for Pro39 (Vasiljeva et al., 2001), cathepsin B (Lockwood, 2004) or papain-like protease 2 (PLP2) from Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) (Han et al., 2005). However, the mechanism of Zn^{2+} , Cu^{2+} and Fe^{3+} inhibition is unclear due to the lack of molecular structures available, the diversity and the very low similarity inside

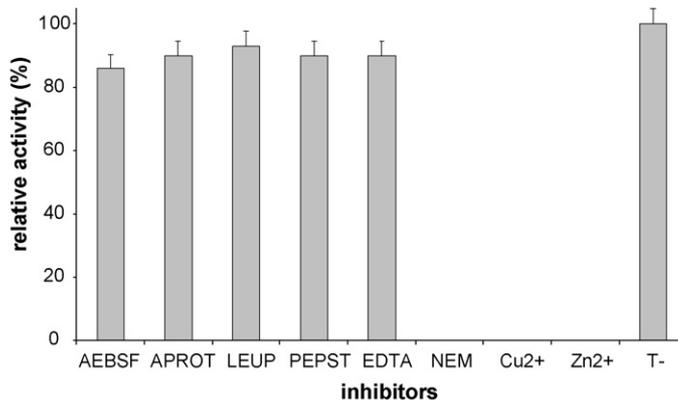


Fig. 3. Inhibitor profile of CHIKV nsP2pro. Several inhibitors were tested for their activity (\pm S.E.) against CHIKV nsP2pro. The test compounds were preincubated with 0.5 μM CHIKV nsP2pro for 10 min at 37 °C followed by the addition of the substrate Boc-AGG-MCA at 100 μM . The different inhibitors tested were, respectively AEBSF (100 μM), aprotinin (10 μM), leupeptin (100 μM), pepstatin A (1 μM), EDTA (10 mM), *N*-ethylmaleimide (NEM, 25 mM), Cu^{2+} (2 mM), Zn^{2+} (2 mM). T-: no inhibitor.

the papain-like cysteine proteases family (Russo and Watowich, 2006). It is interesting to note that zinc and zinc conjugates have been proven effective for inhibiting SARS-CoV replication *in vivo* through simultaneous inhibition of SARS-CoV PLP2 and 3CL proteases (Hsu et al., 2000). As zinc salts (zinc acetate) are already used as therapeutics in humans, they could represent a promising drug for treatment of other viral infections.

The success of retroviral proteases inhibition and the early promise being shown by inhibitors of the NS3 hepatitis C virus (Lin et al., 2004) have demonstrated the feasibility and the interest of antiviral strategies based on protease inhibition. Functional assay such as the one we develop in this work will allow high-throughput screening of chemical libraries in search of inhibitors and should help for further characterization of the nsP2 alphaviruses' protease.

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