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NS3 protease of Langat tick-borne flavivirus cleaves serine protease substrates

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Abstract

Langat (LGT) virus, initially isolated in 1956 from ticks in Malaysia, is a naturally occurring nonpathogenic virus with a very close antigenicity to the highly pathogenic tick-borne encephalitis (TBE) Western subtype virus and TBE Far Eastern subtype virus. NS3, the second largest viral protein of LGT virus, is highly conserved among flaviviruses and contains a characteristic protease moiety (NS3 pro). NS3 pro represents an attractive target for anti-protease molecules against TBE virus. We report herein a purification method specially designed for NS3 pro of LGT using a strategy for proper refolding coupled with the enzymatic characterisation of the protein. Different *p*-nitroanilide substrates, defined on canonic sequences for their susceptibility to Ser-protease, were applied to the proteolytic assays of the protein. The highest values were obtained from substrates containing an Arg or Lys (amino acid) residue at the P1 position. This purification method will facilitate the future development of reliable testing procedures for anti-proteases directed to NS3 proteins.

Keywords: Langat virus; NS3; Serine protease; Tick-borne encephalitis virus; Refolding; *p*-Nitroanilide substrates

Flaviviruses are arthropod-transmitted viruses that belong to the *Flaviviridae* family [1]. The genus *Flavivirus* includes the high human pathogenic agents of yellow fever, dengue, tick-borne encephalitis (TBE), Japanese encephalitis, St. Louis encephalitis and West Nile fever. They are either tick- or mosquito-borne micro-organisms and their close phylogenetic relationships allowed their detection by a consensus hemi-nested PCR [2]. The mammalian tick-borne virus group includes *Gadgets Gully virus*, *Kadam virus*, *Kyasanur Forest disease virus*, *Omsk haemorrhagic fever virus*, *Royal Farm virus*, and *Karshi virus* [3]. Within this group, *Louping ill virus*, *Powassan virus*, *Langat virus*, and the three subtypes of *TBE viruses* share the same sero-complex properties [4].

TBE occurs in an endemic pattern over a wide area of Europe and the former Soviet Union corresponding to the distribution of its tick vector and the annual incidence is several thousands of cases in humans [5,6]. There is no specific treatment and the fatality rate is 1–20% depending on the virulence of the strain. Neurologic sequelae occur in 10–60% of survivors.

Langat (LGT) virus, initially isolated in 1956 from ticks in Malaysia [7], is not known to be associated with a naturally occurring human disease and does not appear to be a veterinary pathogen either. LGT virus is a naturally occurring nonpathogenic virus with a very close antigenicity to the highly pathogenic TBE Western subtype virus and the TBE Far Eastern subtype virus, with the same genomic structure [8]. LGT has been recognised by its inducing capacity of cross-reactive neutralising antibodies. For this reason, LGT virus was considered for vaccine development as a live attenuated vaccine for prevention of TBE [8].

LGT virus genome consists of a single-stranded RNA of positive polarity with a type I cap structure at the

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5′-end and encodes a single polyprotein precursor arranged as NH2–C–prM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5–COOH. Maturation of the polyprotein precursor is mediated both co-translationally and post-translationally by host signalases and virus protease within the endoplasmic reticulum, to produce three structural proteins, C (core), prM (precursor to membrane), and E (envelope) and at least seven non-structural (NS) proteins (NS1–NS5) [9,10].

The intracellular NS3 protein, the second largest viral protein (about 70 kDa), is highly conserved among flaviviruses and cleaves proteolytic sites on the non-structural moiety in endoplasmic reticulum. A trypsin-like serine protease moiety of NS3 was identified within the N-terminal 180 amino acid residues by sequence comparisons [11,12] and the enzymatic activity has been confirmed by deletion analysis [13] and site-directed mutagenesis of the residues comprising the proposed catalytic triad or the substrate-binding pocket [14]. Analysis of polyprotein processing in infected cell models [15] established that the NS3 protease moiety (NS3 pro) is expressed as a heterodimeric association with the viral activator protein NS2B. The active protease NS2B-3 cleaves in both *cis* and *trans* configurations at the NS2A–NS2B, NS2B–NS3, NS3–NS4A, and NS4B–NS5 junctions [16,17]. These sites have in common Lys–Arg, Arg–Arg, Arg–Lys, and occasionally Gln–Arg at the P2/P1 positions [18], followed by a short stretch with Gly, Ala, or Ser at the P1′ position [19]. In addition, the viral protease also cleaves internally within NS2A and NS3 [20].

Because the NS3 protein plays a crucial role in the virus life cycle [21,22], the inhibition of viral protease could be a rationale strategy for the treatment of infections with *Flaviviridae* [23], as it was successfully performed in HIV-1 therapy [24]. As the first attempt to develop inhibitory assays of the protease of tick-borne flaviviruses, we have designed a purification procedure with a refolding step, adapted for the recombinant protease moiety of LGT virus with elementary enzymatic parameters using *p*-nitroanilide substrates.

Materials and methods

LGT virus and isolation of viral RNA. LGT virus (TP21 strain) was obtained from mouse brain tissues and propagated in Vero cells as previously described [25]. RNA was extracted using silica gel membrane spin columns (QIAmp Viral RNA 250; Qiagen S.A., Courtaboeuf, France) from 280 μL samples obtained from cell culture supernatant. The extracted nucleic acid was stored at 80 °C and if needed diluted with ultrapure water (pre-treated by diethyl pyrocarbonate, Sigma–Aldrich, St. Quentin Fallavier, France; 1:1000 dilution).

Construction of the expression plasmid. PCR products were generated using RNA of LGT virus (TP 21 strain) as a template prepared above and the oligonucleotide pair: 5′oligo NS3S: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGATGTAAAAAATGGAGTGTACCGC; 3′oligo NS3R: GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGCCAACTACTGACTGTGGGAGATT (sequence of attB site is underlined). The RT-PCR were carried out according to specification of Qiagen one-step RT-PCR Kit (Qiagen S.A.). The PCR thermal cycling incubations were performed as follows: reverse transcription at 50 °C for 30 min, denaturation at 98 °C for 15 min; amplification at 94 °C for 30 s, 56 °C and 72 °C for 1 min each, 40 cycles. All thermal cycling was performed with PE Applied Biosystems 2400 machines. The amplification products were identified by electrophoresis in a 2% agarose gel after staining by ethidium bromide and visualisation under UV light transillumination [26].

The attB–attB fragment of LGT NS3 pro was inserted into the pDEST17 Vector according to specification of Gateway Cloning Technology (Invitrogen–Gibco BRL, Cergy Pontoise, France). RT-PCR amplification and expression plasmid were sequenced; the sequencing reaction was performed by PCR amplification in a final volume of 20 μL using 100 ng PCR products, 5 pmole primer and 8 μL BigDyeTerminators premix according to Applied Biosystems protocol. After heating to 94 °C for 2 min, the reaction was cycled as follows: 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 4 min at 60 °C (9600 thermal cycler Perkin–Elmer). Exclusion columns performed removal of excess of BigDyeTerminators. The samples were dried in a vacuum centrifuge and dissolved with 2 μL deionised formamide EDTA, pH 8.0 (5/1). The samples were loaded onto an Applied Biosystems 373XL sequencer and run for 12 h on a 4.5% denaturing acrylamide gel. The resulting plasmid (pLGTNS3 pro) encodes the 1508–1674 codon sequence, corresponding to the NS3 protease moiety with 6× His residues attached to the N terminus [6] (EMBL/GenBank, Accession No. P29837).

Sequence alignment. Sequences of NS3 protease moiety of some strains of flaviviruses were compared after using the DNASIS software according to the Higgins and Sharp algorithm CLUSTAL 4 [27]. The similarity scores were calculated as the number of exactly matched residues (top diagonals = 1) in the Wilbur and Lipman alignment between two sequences, minus a fixed penalty (=10) for every gap [28]. Floating gap penalty was 10 and *K*-tuple was 1.

Expression, purification and refolding of recombinant NS3 pro. The construction of the LGT NS3 pro was expressed as fusion proteins containing 6× His residues attached to the N terminus for Ni-chelating affinity purification. The *Escherichia coli* strain BL-21 SI (Invitrogen, Gibco BRL), transformed with the expression plasmid pLGTNS3 pro, was grown at 37 °C in LBON (Luria–Bertani growth without NaCl) supplemented with 100 mg mL^{-1} ampicillin to reach $A_{600} = 0.5$, induced with 0.3 M NaCl and shifted to 30 °C for 3 h 30 min. Cells were pelleted by centrifugation at 6000*g* for 10 min at 4 °C and resuspended in the lysis buffer (5 mM 2-mercaptoethanol, 300 mM NaCl, and 50 mM Tris–HCl, pH 8.5). Cells were disrupted by sonication at 2.4 kV and 4 kΩ (6× 10-s bursts) on ice. Bacterial cell lysates were centrifuged at 27,000*g* for 30 min at 4 °C. The 6His–NS3 pro (173 aa) was located in the insoluble fraction. The pellet was then resuspended in 5 mM 2-mercaptoethanol, 2 M NaCl, and 50 mM Tris–HCl, pH 8.5, and clarified by centrifugation at 27,000*g* for 30 min at 4 °C. The pellet was resuspended in a 5 mM 2-mercaptoethanol, 300 mM NaCl, 1% Triton X-100, 50 mM Tris–HCl, pH 8.5, and clarified by the same centrifugation as above. The pellet was solubilised in denaturation buffer (5 mM 2-mercaptoethanol, 300 mM NaCl, 6 M urea, 50 mM Tris–HCl, pH 8.5). A suspension of Ni^{2+} affinity resin (Ni-NTA Superflow, Qiagen S.A.) was pre-equilibrated with denaturation buffer (20× bed volume) and the solubilised fraction (loading) was incubated with the resin overnight at 4 °C. The resin was centrifuged at 1000*g*, and the unbound fraction was removed. The resin with the bound protein was poured into a column (1.6 × 20 cm; Bio-Rad S.A., Marnes-la-coquette, France) and the column was washed with denaturation buffer (20× bed volume).

The refolding of the denatured protein on the column was carried out by an inverse gradient of urea (6–0 M) in the same buffer for 5 h at 0.7 mL min^{-1} and elution was carried out with 0.5 M imidazole in the

Table 1
Cleavage of chromogenic substrates by NS3 pro of LGT virus

p-Nitroanilide substrate	Final concentration of substrate (mM)	LGT NS3 pro[a]	Absorbance[b]	Substrate specific for
N-p-Tosyl-**Gly**–**Pro**–**Arg** *p*-nitroanilide	0.4	0	–	Trypsin
		Negative control	–	
		+	0.439 ± 0.014	
N-Benzoyl-**Pro**–**Phe**–**Arg** *p*-nitroanilide	1	0	–	Thrombin
		Negative control	–	
		+	0.533 ± 0.014	
N-p-Tosyl-**Gly**–**Pro**–**Lys** *p*-nitroanilide	0.6	0	–	Trypsin
		Negative control	–	
		+	0.403 ± 0.016	
*N*α-Benzoyl-L-**Arg** *p*-nitroanilide (BAPNA)	2.5	0	–	Plasmin
		Negative control	–	
		+	0.451 ± 0.010	
Gly–**Phe** *p*-nitroanilide	2	0	–	Cathepsin C
		Negative control	–	
		+	–	

[a] Enzymatic conditions: 0, no enzyme; negative control, nontransformed *E. coli BL21-SI* purified according to the same conditions as LGT NS3 pro; +, 0.5 μM recombinant NS3 pro of LGT virus.
[b] Mean of triplicate absorbance at 405 nm (± standard deviation); –, under the threshold limit ($A_{405} \leqslant 0.08$).

same buffer. Nontransformed *E. coli BL21-SI* lysate was submitted to the same procedure and the corresponding eluate was taken as a negative control.

The aliquots from the peak fractions selected by their protein content (Bio-Rad protein assay, Bio-Rad S.A.) were submitted to SDS–polyacrylamide gel electrophoresis (PAGE) [29]. The peak fractions were then pooled and proteins precipitated using 10% (w/v) trichloroacetic acid. The purity of the enzyme preparation was checked by SDS–PAGE and Western blot using mouse monoclonal penta-His antibody (Qiagen S.A.) (1:1000), followed by a goat anti-mouse peroxidase conjugate (Qiagen S.A.) (10:1000) [26].

Protease assay on chromogenic substrates. NS3 protease activity was assayed overnight at 23 °C on *p*-nitroanilide substrates (Sigma–Aldrich) (Table 1) in 96-well plates with each well containing a final volume of 150 μL, 0.5 μM LGT NS3 pro in the reaction buffer (50 mM NaCl, 5 mM 2-mercaptoethanol, 50 mM Tris, pH 8.5). Assay was monitored at A_{405} using the iEMS reader MF 1401 (Labsystems, Cergy Pontoise, France).

Results and discussion

Sequence alignment of NS3 pro encoded by flavivirus

The N-terminus moiety of NS3 from most of the flavivirus strains exhibited similar proteolytic properties. This prompted us to examine sequence alignment of NS3 pro from 16 related flavivirus strains representing the most human pathogenic viruses. The alignment of these 16 peptide sequences with the 170 residues of the NS3 moiety is presented in Fig. 1. Eighty percentage of the amino acids was conserved between NS3 pro of LGT virus and TBE virus. The positions of catalytic triad residues (His-37, Asp-61, and Ser-122, numbered according to the LGT virus sequence numeration) were conserved among these 16 flavivirus strains. About 27% of the amino acids were conserved among flavivirus protease moieties, with the majority located around the active site.

Expression and purification of the active LGT protease moiety

The recombinant LGT protease was produced in bacterial system using the *E. coli BL21-SI* strain transformed by the plasmid pLGTNS3 pro. The purification of the recombinant NS3 pro was carried out from the bacterial lysate using a Ni^{2+} affinity matrix and denaturation and refolding steps. The LGT NS3 pro protein was identified by SDS–PAGE as a 21 kDa band (Fig. 2A) with a hexahistidine tag (Fig. 2B). A 1-liter culture yielded 0.5 mg protein. However, the expressed protein was found to be associated with inclusion bodies. This required subsequent denaturation and refolding using high salt concentration to minimise protein–protein interactions and Triton X-100 to dissociate protein–membrane aggregates, for achievement of purification of a soluble and active enzyme (Fig. 3A). The purity of the preparation was estimated as over 95%, as judged by SDS–gel electrophoresis (Fig. 3B).

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Flavivirus strains       10         20         30       # 40         50
LGT              -DVKNGVYRI YTPGLLWGQR QIGVGYGAKG VLHTMWHVTR GAALLVDGVA
TBE              -E.RD..... FS........ .V.....F.. .......... ....SINDAV
TBE Vasilchenko  -E.RD..... FS........ .V.....F.. .......... ....SINDAV
TBE Hypr         -E..D..... FS...F...N .V.....S.. .......... ....SI.DAV
LI               -E..D..... FS...F.... .V.....H.. .......... ....SI.DAV
POW              -.I.E..... .A...F..Y. .......T.. .......... ....S.E.AT
MVE              G.TTP..... MAR.I.-.RY .A...VMHE. .F..L..T.. ...IMSGEGR
JE               G.TTT..... MAR.I.-.TY .A...VMYEN .F..L..T.. ...IMSGEGK
WN               G.TTT..... M.R...-.SY .A.A.VMVE. .F..L..T.K ....MSGEGR
WN NY99          G.TTT..... M.R...-.SY .A.A.VMVE. .F..L..T.K ....MSGEGR
DEN1             AVLDD.I... LQR...-.RS .V...VFQE. .F........ ..V.MYQ.KR
DEN2             AELED.A... KQK.I.-.YS ...A.VYKE. TF........ ..V.MHK.KR
DEN3             AELEE..... KQQ.IF-.KT .V...VQKE. .F........ ..V.THN.KR
DEN4             AALSE..... MQR..F-.KT .V...IHME. .F........ .SVICHETGR
YF 17DD          EHLED.I.G. FQSTF.-.AS .R...VAQG. .F........ ..F.VRN.KK
YF FNV           EHLED.I.G. FQSTF.-.AS .R...VAQG. .F........ ..F.VRN.KK

                        60 #       70         80          90         100
LGT              VGPYWADVRE DVVCYGGAWS LESRWRG-ET VQVHAFPPGR AHETHQCQPG
TBE              A.......K. .......... ..EK.K.-.. .......... ...V......
TBE Vasilchenko  A.......K. .......... ..EK.K.-.. .......... ...V......
TBE Hypr         A......... .......... ..EK.K.-.. .........K ...V......
LI               A.......K. .......... ..EK.K.-.. .......... ...V......
POW              S......... .........G .DKK.G.-.V ........DS G.KI......
MVE              LT...GN.K. .R.T...P.K .DQK.N.VDD ..MIVVE..K PAINV.TK..
JE               LT...GS.K. .RIA...P.R FDRK.N.TDD ...IVVE..K .AVNI.TK..
WN               LD...GS.K. .RL....P.K .QHK.N.HDE ..MIVVE..K NVKNV.TK..
WN NY99          LD...GS.K. .RL....P.K .OHK.N.HDE ..MIVVE..K NVKNV.TK..
DEN1             LE.S..S.KK .LIS...G.R FQGS.NAG.E ...I.VE..K NPKNV.TA..
DEN2             IE.S....KK .LIS...G.K ..GE.KEG.E ...L.LE..K NPRAV.TK..
DEN3             LE.N..S.KK .LIS...G.R .SAQ.QKG.E ...I.VE..K NPKNF.TM..
DEN4             LE.S.....N .MIS...G.R .GDK.DKE.D ...L.IE.RK NPKHV.TK.S
YF 17DD          LI.S..S.K. .L.A...S.K ..G..D.E.E ..LI.AV..K NVVNV.TK.S
YF FNV           LI.S..S.K. .L.A...S.K ..G..D.E.E ..LI.AV..K NVVNV.TK.S

                       110        120  #     130        140        150
LGT              ELILENGRKM GAIPIDLAKG TSGSPIMNSQ GEVVGLYGNG -LKTNDTYVS
TBE              ..L.DT..R. ..V....... ......L.A. .A........ -....E....
TBE Vasilchenko  ..L.DT..R. ..V....... ......L.A. .A........ -....E....
TBE Hypr         ....DT...L .......V.. ......L.A. .V........ -....E....
LI               ....DT.KRL .......... ......L.A. .V........ -P...ES...
POW              K.N..G..VL .......PR. ......I.A. .D.L...... -..S..V.I.
MVE              IFKTAH.-EI ..VSL.YPI. ......V..N ..II...... VILG.GA...
JE               VFRTPF.-EV ..VSL.YPR. ......LDFN .DII...... VELGDGS...
WN               VFKTPE.-EI ..VTL.YPT. ......VDKN .D.I...... VIMP.GS.I.
WN NY99          VFKTPE.-EI ..VTL.FPT. ......VDKN .D.I...... VIMP.GS.I.
DEN1             TFKTPE.-EV ...AL.FKP. ......V.RE .KI....... VVT.SG....
DEN2             LFRTNT.-TI ..VSL.FSP. ......VDKK .K........ VVTRSGA...
DEN3             IFQTTT.-EI ...AL.FKP. ......I.RE .K........ VVTK.GG...
DEN4             LFKTLT.-EI ..VTL.FKP. ......I.RK .K.I...... VVTKSGD...
YF 17DD          LFKVR..GEI ..VAL.YPS. ......V.RN ...I...... I.VGDNSF..
YF FNV           LFKVR..GEI ..VAL.YPS. ......V.RN ...I...... I.VGDNSF..

                       160        170
LGT              SIAQGEVEKS RPNLPQSVVG
TBE              .......... ......A...
TBE Vasilchenko  .......... ......A...
TBE Hypr         ......A... ......A...
LI               ......A... ......A...
POW              .....N.... ..EM.LA.Q.
MVE              A.V...R-VE E.VPEAYNPE
JE               A.V..DR-QE E.VPEAYTPT
WN               A.V...R-ME E.APAGFEPE
WN NY99          A.V...R-MD E.IPAGFEPE
DEN1             A...AKASQE G.LPEIEDE-
DEN2             A...T.KSIE DN-PEIEDDI
DEN3             G...TNA.PD G.TPELEEE-
DEN4             A.T.A.R-IG E.DYEVDEDI
YF 17DD          A.S.T..KEE GK--EELREI
YF FNV           A.S.T..KEE GK--EELQEI
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Fig. 1. Sequence alignment of NS3 protease moieties of flavivirus strains representative of pathogenic viruses. Numbering is according to the polyprotein of LGT virus (TP21 strain) and to the N-terminus of NS3 protease moiety (1508–1674). The catalytic triad residues are marked with hashes (#). Gaps (which can be interpreted as base deletions) are indicated by hyphens (–). Residues conserved among the flaviviruses sequences are shown in bold. The sequences were obtained from EMBL/GenBank, Accession Nos.: Langat virus (LGT): P29837; tick-borne encephalitis virus (TBE): AAF82240; TBE *Vasilchenko*: AF069066; TBE *Hypr*: U39292; Louping ill virus (LI): CAA69190; Powassan virus (POW): A46105; Murray valley encephalitis (MVE): NC000943; Japanese encephalitis (JE): L48961; West Nile virus (WN): NC001563; WN *NY99*: AF196835; dengue virus type 1 (DEN1): NC001477; DEN2: NC001474; DEN3: NC001475; DEN4: P09866; yellow fever virus (YF) *17DD*: U17066; YF *FNV*: U21055.

This purification procedure is consistent with Yusof et al. [20] who reported a similar strategy for expression of NS3 pro of dengue-2 virus and noted that the protein of interest was found to be associated with inclusion bodies. Purification was first achieved after denaturation in the presence of urea and then refolding of the

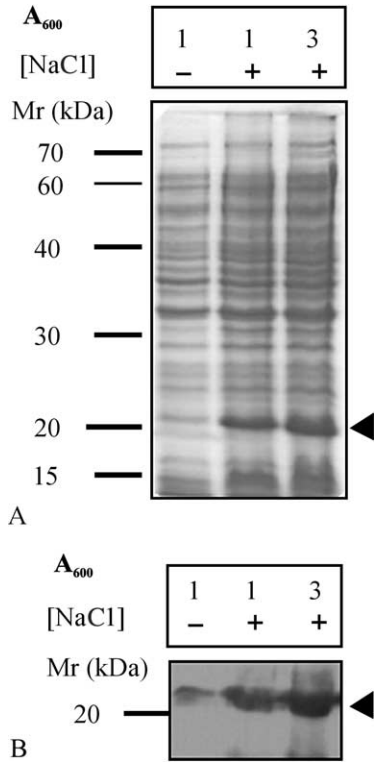

Fig. 2. Expression assays of LGT NS3 pro. (A) Analysis of the expression of NS3 pro. Twenty μL cell homogenate prepared from culture with $A_{600} = 1$ or $A_{600} = 3$ was submitted to SDS–12.5% PAGE and bands were stained by Coomassie blue. (+) Homogenate from NaCl-induced cells; (−) negative control, homogenate from transformed cells but not induced. (B) Immunoblot analysis. The homogenate from cell culture was transferred to a PVDF membrane and submitted to Western blot analysis. Lanes 1–3 were probed with anti-penta-His antibody. The arrowheads indicate the position of NS3 pro.

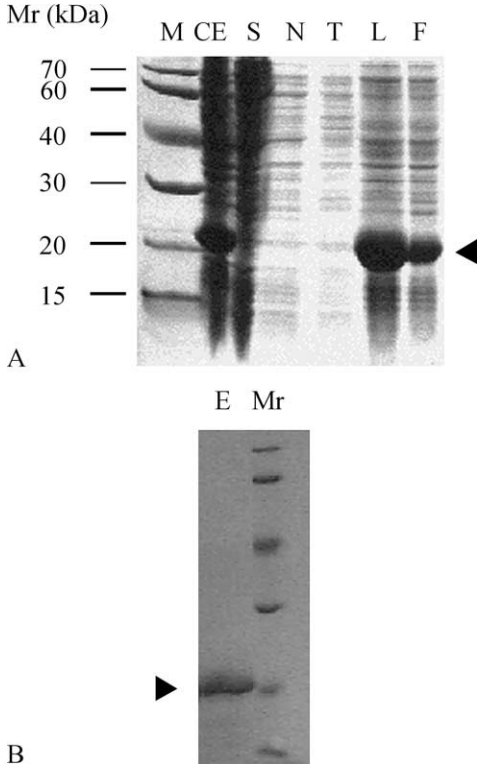

Fig. 3. Purification of LGT NS3 pro. (A) Selective protein extraction by high salt concentration and detergent. Proteins were separated by SDS–12.5% PAGE stained with Coomassie blue. CE: crude extract; S: soluble fraction; N: NaCl fraction; T: Triton fraction; L: loading (solubilised fraction); F: flow-through. (B) Purification of LGT NS3 pro. E: Elution of refolded and purified LGT NS3 pro. The arrowheads indicate the position of NS3 pro.

expressed products by extensive dialysis. However, the refolding dialysis step produced unacceptable levels of precipitation. Instead, we found that the LGT NS3 pro was successfully refolded on the column by a slow inverse gradient of urea [30]. During this renaturation step, the NS3 pro remained bound onto the matrix and the refolded protein was eluted by a gradient of imidazole (Fig. 3B).

LGT NS3 pro cleaves chromogenic substrates

We then investigated whether synthetic substrates could be catalysed in the presence of LGT NS3 pro. A variety of *p*-nitroanilide substrates were selected because they were specific for serine protease such as thrombin, plasmin or trypsin. These *p*-nitroanilide substrates contain either one basic amino acid or no basic amino acid. Chromogenic substrates (Table 1) were incubated with purified NS3 pro of LGT at 23 °C. The absorbance at 405 nm (A_{405}) observed in the case of susceptible substrates was 0.4–0.5 with the highest value for *N*-Benzoyl–Pro–Phe–Arg *p*-nitroanilide (Table 1). Non-transformed *E. coli BL21-SI* was used as negative control with an A_{405} value of less than 0.08.

Table 1 shows that LGT NS3 pro cleaved serine protease substrates containing basic amino acid residues (Arg or Lys) but not the substrate Gly–Phe *p*-nitroanilide containing the residues Gly and Phe and not the substrate specific for the cysteine protease cathepsin C. These results indicate that NS3 pro cleaved serine protease substrates specific for thrombin, plasmin, or trypsin (Table 1) and that, unlike trypsin, NS3 pro of LGT virus has marked preference for basic residues at P1. The substrate conversion was less than 5% for BAPNA and less than 25% for other substrates. This discrepancy could depend on in vitro conditions different from the cell context in endoplasmic reticulum, even when long incubation times are used as previously reported for NS3 pro of dengue-2 virus [31].

Sequences with identification of the cleavage sites of LGT protease and 15 other flaviviral NS3 proteases are shown in Table 2. P2 and P1 residues for NS2B, NS3, NS4A, and NS5 are highly conserved among flaviviruses and contain basic amino acid residues (Arg–Arg, Lys–Arg, or Arg–Lys) or occasionally Gln–Arg (DEN1,

Table 2
Sequences of some flavivirus strains with identification of the internal cleavage sites by their corresponding NS3 serine proteases

Flavivirus	Cleavage sites of flavivirus polyprotein substrates			
	NS2A-NS2B	NS2B-NS3	NS3-NS4A	NS4B-NS5
LGT	ASRG**RR**↓SFNEPM	LGSP**RR**↓TDLVFS	YASG**GR**↓SVGDVL	TTGT**RR**↓GGSEGD
TBE	THRG**RR**↓SFSEPL	MRSA**RR**↓SDLVFS	YASG**RR**↓SIGDVL	ASGG**RR**↓GGAEGD
TBE *Vasilchenko*	THRG**RR**↓SFSEPL	MRSA**RR**↓SDLVFS	YASG**RR**↓SIGDVL	ASGS**RR**↓GGAEGD
TBE *Hypr*	AHRG**RR**↓SFSEPL	LRSS**RR**↓SDLVFS	YASG**RR**↓SFGDVL	ASGG**RR**↓GGSEGD
LI	AHRG**RR**↓SFSEPL	MRSS**RR**↓SDLVYS	YASG**RR**↓SFGDVL	ASGS**RR**↓GGSDGD
POW	GGRG**RR**↓SFSEPL	FSST**RR**↓TDLVFS	YASG**RR**↓SAVDIL	TQGA**RR**↓GGAEGS
MVE	NPNK**KR**↓GWPATE	LKYT**KR**↓GGVFWD	FAAG**KR**↓SAIGFF	KPAF**KR**↓GRAGGR
JE	NPNK**KR**↓GWPATE	LKTT**KR**↓GGVFWD	FAAG**KR**↓SAVSFI	KPSL**KR**↓GRPGGR
WN	DPNR**KR**↓GWPATE	LQYT**KR**↓GGVLWD	FASG**KR**↓SQIGLV	KPGL**KR**↓GGAKGR
WN *NY99*	DPNR**KR**↓GWPATE	LQYT**KR**↓GGVLWD	FASG**KR**↓SQIGLI	KPGL**KR**↓GGAKGR
DEN1	KIWG**RK**↓SWPLNE	QKKK**QR**↓SGVLWD	FAAG**RR**↓SVSGDL	LGGG**RR**↓GTGAQG
DEN2	RTSK**KR**↓SWPLNE	EVKK**QR**↓AGVLWD	FAAG**RK**↓SLTLNL	TTST**RR**↓GTGNIG
DEN3	DTLK**RR**↓SWPLNE	QKQT**QR**↓SGVLWD	FAAG**RK**↓SIALDL	VGTG**KR**↓GTGSQG
DEN4	KGAS**RR**↓SWPLNE	QVKT**QR**↓SGALWD	FASG**RK**↓SITLDI	AQTP**RR**↓GTGTTG
YF *17DD*	RIFG**RR**↓SIPVNE	VRGA**RR**↓SGDVLW	FAEG**RR**↓GAAEVL	MKTG**RR**↓GSANGK
YF *FNV*	RIFG**RR**↓SIPVNE	VRGA**RR**↓SGDVLW	FAEG**RR**↓GAAEVL	MKTG**RR**↓GTANGK

Note. The sequences were obtained from the EMBL/GenBank databases. For accession numbers and acronyms of viruses see legend of Fig. 1.

DEN2, DEN3, and DEN4 for NS2B–NS3 cleavage site) or Gly–Arg (LGT for NS3–NS4A cleavage site). They are usually flanked by short side-chain amino acids, most commonly Gly, Ser, Ala, Thr, or Leu (Table 2).

NS3 pro of dengue-2 virus (associated or not with NS2B) was reported to be active towards a chromogenic substrate containing an Arg residue at P1 and a benzoyl moiety instead of a P2 residue [20,31]. We report herein the same cleavage specificity after incubation of NS3 pro of LGT virus with *p*-nitroanilide substrates. This suggests a similar cleavage process between NS3 pro of dengue-2 virus and of LGT virus. The proteolytic mechanism of NS3 pro of tick-borne flaviviruses would not exhibit any difference with the NS3 pro of mosquito-borne flaviviruses.

We report a procedure for convenient expression and purification of the LGT protease in *E. coli*, with characterisation of some chromogenic substrates. In 1993, the catalytic triad of the NS3 protease moiety of tick- borne flavivirus was discovered by site-directed mutagenesis [32], but there is yet no confirmation of its specific serine protease activity. We now show that LGT NS3 pro cleaves serine protease substrates with strong similarity to NS3 pro of dengue-2 virus including critical and unusual requirement for basic residues such as Arg or Lys at P1/P′1. These results will allow us to establish reliable screening activity test for viral protease inhibitors against NS3 pro of LGT virus as well as their lack of inactivation capacity towards cellular proteases essential for physiological function. An alternative strategy using serpins could be tested, following the ability of host serpins to control the virus serine protease NS3 of Hepatitis C virus [33]. Based on the high homology observed between LGT and TBE NS3 sequences, the selected drugs are attractive candidates for control testing experiments on pathogenic strains of TBE virus.

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