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Expression, purification, and in vitro activity of an arterivirus main proteinase

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Abstract

To allow the biochemical and structural characterization of the chymotrypsin-like “main proteinase” (non-structural protein 4; nsp4) of the arterivirus prototype Equine Arteritis Virus (EAV), we developed protocols for the large-scale production of recombinant nsp4 in *Escherichia coli*. The nsp4 proteinase was expressed either fused to maltose binding protein or carrying a C-terminal hexahistidine tag. Following purification, the nsp4 moiety of MBP-nsp4 was successfully used for structural studies [Barrette-Ng, I.H., Ng, K.K.S., Mark, B.L., van Aken, D., Cherney, M.M., Garen, C., Kolodenco, Y., Gorbalenya, A.E., Snijder, E.J., James, M.N.G., 2002. Structure of arterivirus nsp4—the smallest chymotrypsin-like proteinase with an alpha/beta C-terminal extension and alternate conformations of the oxyanion hole. *J. Biol. Chem.* 277, 39960–39966]. Furthermore, both forms of the EAV proteinase were shown to be proteolytically active in two different *trans*-cleavage assays. Recombinant nsp4 cleaved the cognate nsp6/7- and nsp7/8 site in in vitro synthesized substrates. In a synthetic peptide-based activity assay, the potential of the recombinant proteinase to cleave peptides mimicking the P9–P7' residues of six nsp4 cleavage sites was investigated. The peptide representing the EAV nsp7/8 junction was used to optimize the reaction conditions (pH 7.5, 25 mM NaCl, 30% glycerol at 30 °C), which resulted in a maximum turnover of 15% of this substrate in 4 h, using a substrate to enzyme molar ratio of 24:1. The assays described in this study can be used for a more extensive biochemical characterization of the EAV main proteinase, including studies aiming to identify inhibitors of proteolytic activity.

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1. Introduction

Many viruses with a single-stranded, positive-sense RNA genome regulate their genome expression by synthesizing large precursor polypeptides (or polyproteins) that are subsequently subjected to limited proteolysis to produce functional viral proteins (for reviews, see Dougherty and Semler, 1993; Gorbalenya and Snijder, 1996; Krausslich and Wimmer, 1988; Spall et al., 1997). RNA virus polyproteins that include replicative proteins are often processed autocatalytically, although in some virus groups cellular proteinases are also involved. In this manner, the expression of the non-structural proteins (or “replicase”

complex) can be regulated in time and space, e.g. to produce alternative cleavage products or stable processing intermediates with unique functions (de Groot et al., 1990; Jore et al., 1988; Lemm et al., 1994; Ypma-Wong et al., 1988).

Viruses in the order *Nidovirales*, which unifies the enveloped, positive-stranded *Arteriviridae*, *Coronaviridae*, and *Roniviridae* (Snijder et al., 2005; Spaan et al., 2005), have a similar polycistronic genome organization, share a conserved array of homologous replicase domains, and use common transcriptional and (post)-translational strategies to regulate their genome expression. Among these strategies, one of the most crucial is the proteolytic maturation of the replicase polyproteins pp1a and pp1ab that are translated from the incoming genome (den Boon et al., 1991; Ziebuhr et al., 2000).

Equine arteritis virus (EAV) is the prototype of the arterivirus family and has a genome of approximately 12.7 kb, of which

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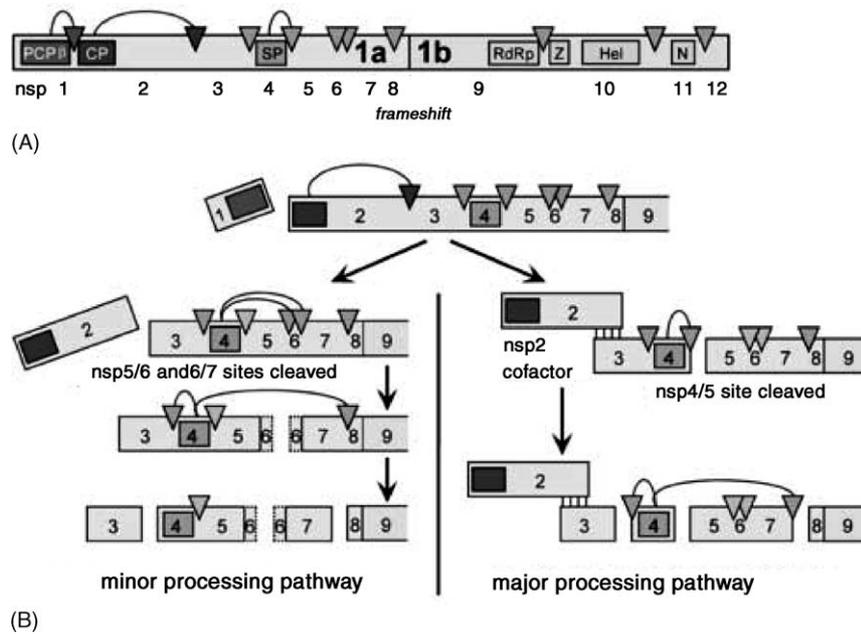


Fig. 1. Proteolytic processing of the EAV replicase. (A) Processing map of the 3175-amino acid EAV replicase polyprotein pp1ab. The three EAV proteinases (PCP β , CP and SP), their cleavage sites and the EAV nsp nomenclature are depicted. PCP β , nsp1 papain-like Cys proteinase; CP, nsp2 Cys proteinase; SP, nsp4 Ser proteinase; RdRp, RNA-dependent RNA polymerase; Z, zinc finger; Hel, helicase; N, nidovirus-specific endoribonuclease (NendoU). (B) Overview of the two alternative processing pathways that apply to EAV pp1a (Wassenaar et al., 1997). The association of cleaved nsp2 with nsp3–8 (and probably also with nsp3–12) was shown to be a cofactor in the cleavage of the nsp4/5 site by the nsp4 proteinase (major pathway). Alternatively, in the absence of nsp2, the nsp5/6 and nsp6/7 sites are processed and the nsp4/5 junction remains uncleaved (minor pathway). The status of the small nsp6 subunit (fully cleaved or partially associated with nsp5 and/or nsp7) remains to be elucidated. Adapted from Barrette-Ng et al., 2002.

the 5'-proximal three quarters contain the replicase gene. The 3'-proximal region of the genome includes seven open reading frames encoding the viral structural proteins, which are expressed from a nested set of subgenomic mRNAs (de Vries et al., 1990; Snijder and Meulenberg, 2001). Replicase gene expression ultimately yields (at least) 12 non-structural proteins (Fig. 1A; Snijder et al., 1994; van Dinten et al., 1996; Wassenaar et al., 1997), which are produced from two primary genome translation products, the large polyproteins pp1a (1728 amino acids) and pp1ab (3175 amino acids), with the latter being expressed following a ribosomal frameshifting event (den Boon et al., 1991). The replicase subunits are released from the polyproteins by three virus-encoded proteinases (Fig. 1), of which the one located in nsp4 is responsible for the processing of the polypeptides that remain after nsp1 and nsp2 have been autocatalytically released from pp1a and pp1ab (Snijder et al., 1992, 1994, 1995, 1996; van Dinten et al., 1999; Wassenaar et al., 1997). Since nsp4 controls the production of the viral RNA-dependent RNA polymerase and RNA helicase, it has been termed the EAV "main proteinase" (Gorbalenya et al., 1991; Ziebuhr et al., 2000).

The arterivirus nsp4 main proteinase belongs to the 3C-like serine proteinases, a distinct group of viral chymotrypsin-like proteolytic enzymes (Barrette-Ng et al., 2002; Snijder et al., 1996; Ziebuhr et al., 2000). The arterivirus proteinase combines the catalytic triad His/Asp/Ser of canonical chymotrypsin-like proteinases with the substrate specificity of the 3C-like cysteine proteinases, a subgroup of chymotrypsin-like enzymes named after the picornavirus 3C proteinases. In the cleaved, 204-residue

EAV nsp4 (which equals Gly-1065 to Glu-1268 of the EAV replicase polyproteins) the catalytic triad is formed by His-39, Asp-65, and Ser-120. Cleavage sites recognized by the nsp4 proteinase carry a Glu at the P 1 position (Gln in one case) and a small amino acid (Gly, Ser, Ala) at the P 1' position (using the cleavage site nomenclature of Schechter and Berger, 1967). Furthermore, nsp4 possesses a unique C-terminal domain (CTD) extension of unknown function, which is not found in most other chymotrypsin-like proteinases and might be involved in modulation of nsp4 activity (Barrette-Ng et al., 2002).

Arterivirus replication depends on the regulation of replicase gene expression in time and space, in which nsp4 plays a key role. Notably, an elegant (presumably) regulatory mechanism connected to nsp4-driven proteolysis was documented in the form of the differential processing of the nsp4–8 part of the EAV replicase polyproteins (Fig. 1B; Wassenaar et al., 1997). Together, the two pathways yield a variety of products, whose role in the viral life cycle remains to be studied in detail.

The biochemical characterization of the arterivirus main proteinase requires the large-scale expression and purification of an active form of the enzyme. In this paper, we describe the production of active, recombinant EAV nsp4 from *Escherichia coli* (*E. coli*), allowing the design of an in vitro cleavage assay, which was used to characterize the properties of nsp4 and its interaction with substrates. The expression system also formed the basis for structural studies, which led to the elucidation of the three-dimensional structure of EAV nsp4 by X-ray crystallography (Barrette-Ng et al., 2002). Together, these studies are a major step towards dissecting the structure–function relation-

ships of this key arterivirus enzyme and the future design of inhibitor drugs.

2. Materials and methods

2.1. Expression plasmid construction

Expression plasmids pMalTnsp4 and pET-nsp4His were constructed using standard recombinant DNA techniques and sequenced. The pMalT2i vector, a derivative of expression plasmid pMal-c2 (New England Biolabs; Wassenaar and Snijder, unpublished data) uses the strong *tac* promoter and the *malE* translation initiation signals to give high-level expression of the cloned sequences. Target genes in the pET plasmid are under control of the strong bacteriophage T7 promoter. (i) Construction of pMalTnsp4. A *NcoI*–*XhoI* restriction fragment

from plasmid pL3440, encoding EAV nsp4 (Wassenaar et al., 1997), was cloned into plasmid pBIN+ (Molenkamp, 2000). The resulting plasmid (pL3440i) was digested with *Bam*HI and *Xho*I and the desired fragment was cloned between the unique *Bam*HI and *Sal*I restriction sites of pMalT2i. This plasmid (pMalTnsp4) encoded the EAV nsp4 proteinase fused to the C-terminus of the *E. coli* maltose binding protein (MBP), with a (Asn)₁₀ spacer and a thrombin cleavage site (Leu–Val–Pro–Arg/Gly–Ser) separating the MBP and nsp4 moieties of the fusion protein, which will be further referred to as MBP-nsp4. Due to the cloning strategy, MBP-nsp4 lacked Glu-204 (nsp4 numbering) and contained three additional amino acids at its C-terminus (Leu–Ala–Ser; Fig. 2C). After digestion with thrombin, also the N-terminus of the protein carried three foreign amino acids (Gly–Ser–Met; Fig. 2C). (ii) Construction of pET-nsp4His. pL3440-E1268P (Wassenaar et al., 1997)

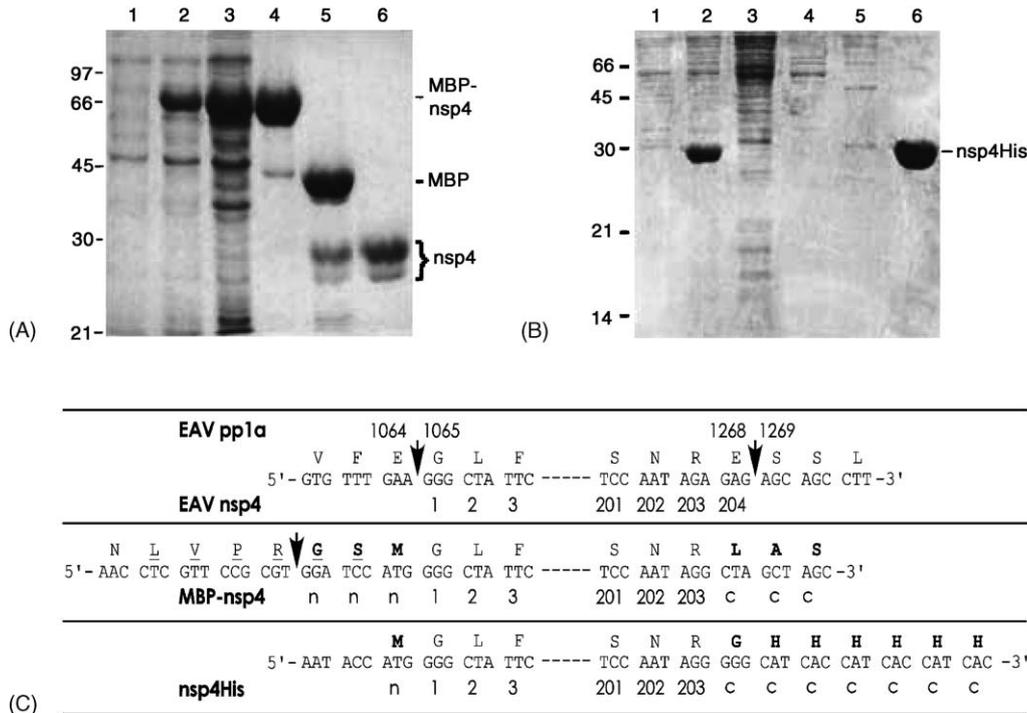


Fig. 2. Expression and purification of recombinant EAV nsp4. (A) Expression, purification, and cleavage of MBP-nsp4. Lanes 1 and 2 show samples of total lysates from non-induced (lane 1) and IPTG-induced (lane 2) cultures of *E. coli* BL21(DE3) cells transformed with pMalTnsp4. After cell lysis and ultracentrifugation (100,000 × *g*) the S100 fraction (lane 3) was loaded on an amylose affinity column and bound protein was eluted with buffer A supplemented with 10 mM maltose (lane 4). After an overnight incubation at 4 °C with 1 unit of thrombin per mg of MBP-nsp4 (lane 5), cleaved nsp4 was further purified by gel filtration (Sephadex G-75), anion exchange chromatography, and repeated loading of the sample on an amylose column until a purity of more than 90% was reached (lane 6). Although mass-spectrometry indicated that the purified nsp4 was homogeneous, the sample migrated as a double band, a repeatedly observed phenomenon with nsp4. Proteins were separated by SDS-PAGE on a 10% polyacrylamide gel, which was stained with Coomassie brilliant blue R-250. (B) Expression and purification of nsp4His. Samples were total lysates from non-induced (lane 1) and IPTG-induced (lane 2) cultures of *E. coli* BL21(DE3) cells transformed with pET-nsp4His or fractions made during purification (lanes 3–6). After cell lysis and ultracentrifugation, the S100 fraction was loaded on a Talon affinity column (Clontech) charged with cobalt chloride. Samples were taken from the flow-through (lane 3), after washing the column with buffer D (lane 4), and with buffer D supplemented with 10 mM imidazole (lane 5). The bound nsp4His was eluted from the column with buffer E (lane 6). For buffer composition see Section 2. As judged from the gel, the purity of the sample was more than 90%. (C) Nucleotide- and amino acid sequences corresponding to the termini of the nsp4 gene in the MBP-nsp4 and nsp4His expression constructs. Foreign amino acids are shown in bold face and indicated with “n” or “c”. The top panel shows N- and C-termini of wild-type nsp4, which is produced from the EAV replicase polyproteins by autoproteolysis of the nsp3/4 and nsp4/5 sites (indicated with ↓). Both pp1a and nsp4 amino acid numbers are given. The middle panel shows the MBP-nsp4 fusion protein. After cleavage by thrombin, compared to native EAV nsp4, three foreign amino acids (Gly–Ser–Met) remain at the N-terminus. The amino acid sequence recognized by thrombin is underlined and the position of the thrombin cleavage site is indicated (↓). At the C-terminus of MBP-nsp4, due to the cloning strategy, the native Glu-204 was replaced by a Leu residue and two additional amino acids were introduced (Ala–Ser). The bottom panel shows the nsp4His protein. A translation initiation codon was added, resulting in an N-terminal foreign Met residue upstream of the native P 1' Gly residue. At the C-terminus of nsp4His, the native Glu-204 was replaced by a Gly residue, which is followed by the (His)₆ tag.

was digested with restriction enzymes *NcoI* and *StuI* and the desired fragment was cloned into a *NcoI* and *BamHI*-digested pET34h vector (Wassenaar and Snijder, unpublished data) together with a *BamHI*-digested Gly-(His)₆-encoding linker (5'-GGGCATCACCATCACCATCACTAAGGGCTGCAGCC-AAGCTGACGGATCC-3'). The resulting plasmid encoded nsp4 with an additional Met at its N-terminus and a C-terminal (His)₆ tag (Fig. 2C). As a precaution, Glu-204 (the P 1 residue of the nsp4/5 cleavage site) was substituted with Gly to ensure that the (His)₆ tag would not be cleaved off as a result of nsp4 activity. The resulting protein will be further referred to as nsp4His. The active site mutation Ser-120 to Ile, which was documented to inactivate the nsp4 proteinase (Snijder et al., 1996), was transferred to pET-nsp4His and the mutant protein (nsp4His-S120I) was used as a negative control in the activity assays. (iii) Construction of pLnspp6–8 and pLnspp6–7. pLnspp6–8 was constructed by self ligation of a *NcoI* and *SmaI* digested pL1aE1451P plasmid (Snijder et al., 1996) of which the *NcoI* site was filled in using the 5' → 3' polymerase activity of the large (Klenow) fragment of DNA polymerase I. pLnspp6–7 was a derivative of pLnspp6–8, in which the sequence (5'-TATGAAGGCCTA-3') encoding the P 2–P 2' residues of the nsp7/8 cleavage site was replaced (by 5'-CTAGCTAGCTAGA-3'), changing Tyr–Glu–Gly–Leu to Leu–Ala–Ser–Stop.

2.1.1. Expression and purification of EAV MBP-nsp4

E. coli strain BL21(DE3), which carries an IPTG-inducible T7 RNA polymerase gene in its chromosome, was transformed with the expression plasmid and grown overnight in 3 ml Luria-Bertani liquid medium supplemented with 100 µg/ml of ampicillin (LBA). Subsequently, 1 ml of the overnight culture was used to inoculate a 3-l flask containing 1 l of LBA. Cultures were grown at 37 °C to an OD₆₀₀ of 0.7 and subsequently protein expression was induced by adding 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were harvested 5 h after induction by centrifugation at 8000 × g for 15 min and cell pellets were stored at –80 °C or directly used for further processing. During the purification procedure, samples and buffers were kept at 4 °C. The pelleted cells were resuspended in buffer A (20 mM Tris pH 8.1, 150 mM NaCl, 1 mM EDTA) and lysed by passing them twice through a 40 K French pressure cell (SLM-Aminco) at 1000 psi (6.9 MPa). Subsequently, the lysate was centrifuged at 100,000 × g for 30 min, resulting in a supernatant (S100) and a pellet (P100) fraction. The S100 fraction was loaded onto an amylose affinity column (New England Biolabs), which was washed with buffer A until the OD₂₈₀ of the flow-through dropped below 0.05. Subsequently, the bound fusion protein was eluted from the column in 3-ml fractions using buffer A supplemented with 10 mM maltose. Fractions containing the MBP-nsp4 fusion protein (as determined by SDS-PAGE analysis) were pooled and the concentration of the fusion protein was determined using the Bradford protein assay (Biorad). Generally, 1 l of culture yielded about 50 mg of fusion protein. Fusion protein-containing fractions were diluted to a concentration of 1 mg/ml and incubated for 16–18 h at 4 °C with 1 unit of thrombin (from human plasma; Sigma) per mg of fusion protein. After

dialysis against buffer B (20 mM Tris pH 8.1, 10 mM NaCl, 1 mM EDTA) at 4 °C, the cleaved fusion protein was loaded onto an anion exchange column (Mono Q, HR 5/5, Pharmacia) and washed extensively with buffer B to remove traces of maltose and contaminating proteins. Bound proteins were eluted from the column in 3-ml fractions using a 50 ml gradient of 10–500 mM NaCl in 20 mM Tris pH 8.1 and 1 mM EDTA. Nsp4-containing fractions were pooled, concentrated (10 mg/ml) and 1 ml fractions were loaded onto a Sephadex G-75 gel filtration column (total bed volume 210 ml). Fractions from this column containing nsp4 were repeatedly loaded onto an amylose affinity column, until all detectable traces of MBP, as judged from SDS-PAGE and Coomassie Brilliant Blue R-250 staining, had been removed. Nsp4 preparations were diluted in buffer B to a concentration of 1–5 mg/ml and stored at –80 °C. The purity and integrity of the nsp4 samples were confirmed by mass spectrometry.

2.1.2. Expression and purification of EAV nsp4His

E. coli strain BL21(DE3) was transformed with expression plasmid pET-nsp4His or pET-nsp4His-S120I and a single colony was incubated in 1 ml LBA at 37 °C until the culture became turbid (approximately 3 h). Subsequently, 50 µl of the culture was plated on a LBA agar plate and incubated overnight at 37 °C. The plate was sliced into small pieces and added to a 2-l flask containing 500 ml of LBA. This culture was grown to an OD₆₀₀ of 0.7 and subsequently protein expression was induced with 1 mM of IPTG. Bacteria were harvested 5 h after induction by centrifugation at 8000 × g for 15 min at 4 °C. Cell pellets were either stored at –80 °C or immediately used for further processing. During the purification procedure, samples and buffers were kept at 4 °C. Cells were resuspended in buffer C (50 mM phosphate pH 8.1, 500 mM NaCl), lysed and centrifuged using the same method as described above for the purification of MBP-nsp4. The S100 fraction was loaded onto a Talon metal affinity resin (Clontech) column (total bed volume 10 ml) and the column was washed with buffer C until the OD₂₈₀ of the flow-through dropped below 0.05. Subsequently, the column was washed with 10 column volumes of buffer D (20 mM Tris pH 8.1, 150 mM NaCl), and 10 column volumes of buffer D supplemented with 10 mM imidazole, to wash off non-specifically bound proteins. Nsp4His was eluted from the column with buffer E (5 mM Tris pH 8.1, 25 mM NaCl, 100 mM imidazole). Nsp4His was more than 90% pure as judged from a Coomassie Brilliant Blue R-250 stained protein gel and generally a yield of 15 mg nsp4His per 1-l culture was obtained. Nsp4His preparations were diluted with buffer G (5 mM Tris pH 8.1, 25 mM NaCl) to a concentration of 1–5 mg/ml and stored at –80 °C.

2.2. Cleavage assay using substrates synthesized in rabbit reticulocyte lysates

Using the TnT system (Promega), plasmids pLnspp6–7 and pLnspp6–8, encoding EAV nsp6–7 and nsp6–8, respectively, were in vitro transcribed and the resulting RNA was translated in the presence of [³⁵S] methionine (Amersham). Typically, transcription/translation reactions with a volume of 25 µl

were carried out for 90 min at 30 °C. The reaction was stopped by adding 1/10 volume of 10× stopmix (20 mM methionine, 5 mg/ml cycloheximide (Sigma), 1 µg/ml RNase A) and 3 µl of the reaction was incubated with 5 µg of either purified nsp4His, MBP-nsp4, or non-purified cleaved MBP-nsp4 in 10 mM Tris pH 8.1 containing 150 mM NaCl, in a total volume of 15 µl. Samples of 3 µl were taken at regular intervals up to 120 min after addition of the proteinase and reaction products were analyzed on 15% tricine gels. As a negative control, 5 µg of purified nsp4His-S120I was used as enzyme in the reaction.

2.3. Peptide synthesis

Peptide substrates were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (SyroII, Multi-SynTech, Witten, Germany). The purity of the peptides was determined by analytical reversed-phase HPLC and proved to be at least 80%. The identity and homogeneity of the peptides was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and analytical reversed-phase chromatography.

2.4. Nsp4 cleavage assay with synthetic peptide substrates

16-mer peptides (representing the P9–P7' residues of a cleavage site) were synthesized by solid-phase chemistry (Table 1). Each peptide was dissolved in 100% DMSO at a concentration of 100 µg/µl and stored at –20 °C. The nsp4 activity assay was performed in a reaction volume of 100 µl, using 25 µl of nsp4 (0.8 µg/µl) and 0.2 µl peptide (100 µg/µl) in the appropriate buffer to yield a final concentration of 0.2 µg/µl (~10 µM) nsp4 and 0.2 µg/µl (~100 µM) peptide in the assay. Cleavage reactions were routinely incubated at 37 °C for 0.5–6 h. The reactions were terminated by the addition of 100 µl of 10% trifluoroacetic acid and stored at –80 °C. Prior to analysis on HPLC, samples were centrifuged for 5 min at 14,000 × g to remove insoluble components. Cleavage products were resolved using a 28 min, 5–90% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The absorbance was determined at 215 nm. Peak areas were calculated by integration.

3. Results

3.1. Expression and purification of MBP-nsp4

The large-scale expression and purification of an MBP-nsp4 fusion protein was achieved using the protocol described in Section 2. Briefly, after induction of expression, large amounts of MBP-nsp4 fusion protein were recovered from the soluble S100 fraction (Fig. 2A) and purification of the protein using an amylose resin affinity column yielded a more than 90% pure fusion protein (as judged from Coomassie Brilliant Blue R-250 stained protein gels). To prevent exposure of the nsp4 proteinase to relatively high temperatures, the release of nsp4 from the MBP moiety by cleavage with thrombin was not performed at the recommended temperature of 25 °C, but at 4 °C. An overnight digestion with 1 unit thrombin per mg of fusion protein was sufficient to cleave virtually all of the fusion protein (Fig. 2A). Nsp4 was further purified by anion exchange chromatography, gel filtration, and repeated loading of the nsp4-containing samples onto an amylose resin affinity column to remove all remaining traces of MBP. Due to the cloning strategy, nsp4 lacked the C-terminal Glu-204 residue (nsp4 numbering) and carried three additional amino acids at its extreme C-terminus (Leu–Ala–Ser). After cleavage by thrombin, also the N-terminus of the proteinase remained attached to three foreign residues (Gly–Ser–Met) (Fig. 2C). Although mass spectrometry indicated that the purified nsp4 sample was homogeneous and of the expected molecular mass, there were two bands visible on the gel (Fig. 2A), which were both recognized by a nsp4-specific antiserum (data not shown). It has been reported before that nsp4, with a predicted molecular mass of 21 kDa, migrates as a 30 kDa protein (Snijder et al., 1996). Presumably, nsp4 is prone to aberrant migration during SDS-PAGE. Nevertheless, the recombinant protein obtained using this protocol was of sufficient purity and homogeneity to be successfully used for crystallography and the subsequent nsp4 structure determination by X-ray crystallography (Barrette-Ng et al., 2002).

3.2. Expression and purification of nsp4His

As alternative to the use of a MBP fusion protein, it was decided to employ the production of a C-terminally (His)₆ tagged protein as a second expression/purification strategy,

Table 1
Sequences of peptide substrates for EAV nsp4 used in this study

Sequence																		
Peptide	Cleavage site	P9	P8	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	P8'
222-34 ^a	nsp5/6	T	F	M	M	K	Y	F	L	E	G	G	V	K	E	S	V	B
222-35 ^a	nsp6/7	A	Y	G	K	P	I	T	Q	E	S	L	T	A	T	L	A	B
222-36 ^a	nsp7/8	Y	V	L	G	K	G	S	Y	E	G	L	D	Q	D	K	V	B
222-37 ^{a,b}	nsp9/10	T	S	F	R	T	K	Q	Y	E	S	A	V	O	T	V	O	B
222-38 ^{a,b}	nsp 10/11	G	P	A	O	G	W	E	K	E	S	N	K	I	S	O	L	B
222-39 ^a	nsp11/12	R	N	A	T	F	Y	V	Q	E	G	V	D	A	V	T	S	B

^a All peptides contained a protective B (amide) at their C-terminus.

^b C (cysteine) residues were replaced by O (2-aminobutyric acid).

which was previously used to successfully purify many proteinases, including several from the 3C-like proteinase group (Chisholm et al., 2001; Someya et al., 2000; Tibbles et al., 1999). Based on the specific affinity of histidine residues for cobalt ions, a convenient one-step purification could be performed using a chelating agarose column charged with cobalt chloride. We reasoned that the (His)₆ tag purification could be more efficient compared to the MBP-mediated purification, as it circumvents the potentially harmful thrombin treatment and does not include the laborious removal of MBP.

The protocol described below essentially followed the original manufacturer's protocol for C-terminally His tagged proteins (described in Section 2). However, some adjustments had to be made regarding the induction of protein expression. When an overnight liquid culture of pET-nsp4His-transformed *E. coli* BL21(DE3) bacteria was used to inoculate a larger culture, induction of protein expression with IPTG was, for unknown reasons, not successful. However, nsp4His synthesis could be induced in cultures grown for shorter periods of time or in colonies picked from freshly transformed plates. Therefore, instead of using an overnight liquid culture as an inoculum, a droplet from a 3 h culture was plated on a LBA plate, which was incubated overnight at 37 °C, sliced into small pieces, and then added to LBA-containing flasks. With this method large cultures could be grown to the right density in a short period of time. After harvesting, lysis, and centrifugation of the cells, the S100 fraction was loaded on a column packed with Talon metal affinity resin (Clontech) charged with cobalt ions and the column was washed extensively with several buffers (see Section 2) to remove contaminating proteins. After the elution of Nsp4His from the column, the protein was more than 90% pure, as judged from a Coomassie Brilliant Blue R-250 stained SDS-PAGE gel, and generally a yield of 15 mg of nsp4His protein per 1-l culture was obtained (Fig. 2B). Active site mutant nsp4His-S120I was purified using the same protocols and yielded comparable quantities of protein. With this expression- and purification protocol, nsp4His could be purified within 8 h, whereas the MBP-nsp4 expression, cleavage and purification protocol would generally take 2 days. The differences between the amino acid sequences at the N- and C-termini of both expression products, and their comparison to the termini of native EAV nsp4, are depicted in Fig. 2C.

3.3. Activity assay with *in vitro* synthesized substrates

To test whether active recombinant proteinases had been isolated, the proteolytic activity of purified MBP-nsp4 and nsp4His was tested in cleavage assays using *in vitro* synthesized substrates as described in Section 2. Substrates represented EAV nsp6–7 and nsp6–8, which are naturally occurring processing intermediates of the minor processing pathway (Wassenaar et al., 1997). Aliquots from *in vitro* translation reactions were incubated with purified recombinant nsp4His or nsp4His-S120I. Samples were taken at 0, 15, 30, 60 and 120 min after addition of proteinase and analyzed on 15% tricine SDS-PAGE gels (Fig. 3). Processing of the two substrates could be easily monitored since cleavage of the nsp6/7, nsp7/8 or both sites resulted

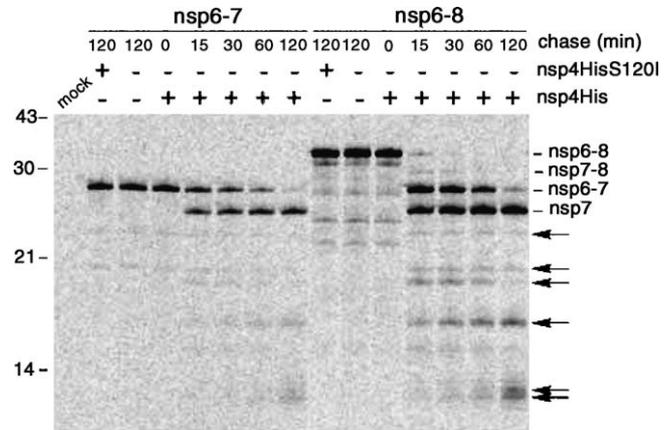


Fig. 3. EAV nsp4His activity in an *in vitro* cleavage assay. Using the TnT system (Promega), expression plasmids encoding EAV nsp6–7 and nsp6–8 were *in vitro* transcribed and the resulting RNA was *in vitro* translated in a rabbit reticulocyte lysate in the presence of [³⁵S] methionine. After a 90-min incubation at 30 °C, transcription and translation were stopped (for details see Section 2). Subsequently, from a typical 25 µl reaction, 3-µl aliquots were taken and incubated with 5 µg of purified nsp4His or nsp4His-S120I (negative control) for up to 120 min in a total reaction volume of 15 µl. Samples were analyzed on a 15% tricine gel and labelled products detected by autoradiography using a Phosphoimager (Biorad). The *in vitro* assay yielded the expected processing products, as predicted from known nsp4 cleavage sites. In addition, a number of smaller cleavage products was detected (indicated with arrows), which may be derived from internal cleavage of nsp7 (see text). Processing products nsp6 and nsp8 were not detected due to their small size of 2.3 and 5.5 kDa, respectively.

in a substrate size reduction of 2.3, 5.5, or 7.8 kDa, respectively. Fig. 3 illustrates that after a 15-min incubation with nsp4His about half of the nsp6–7 substrate had been processed, although the remaining substrate was processed much slower. Similar results regarding the processing of the nsp6/7 site were obtained with the nsp6–8 substrate, but the second cleavage site in this substrate (the nsp7/8 site) was processed much faster. Already after 15 min, almost no nsp6–8 or nsp7–8 could be detected, suggesting that the nsp7/8 site had been cleaved in all of the available substrates (Fig. 3). Surprisingly, processing of each of the substrates produced several additional products (indicated with arrows in Fig. 3). These were not observed after incubation with the inactive control enzyme nsp4His-S120I or when the reaction was performed without enzyme, suggesting that the generation of these products depended on the proteolytic activity of nsp4His. Based on their sizes, these products may have been derived from internal cleavage of nsp7, a possibility that is currently being investigated in more detail.

Using an identical experimental set-up, partially purified cleaved and uncleaved MBP-nsp4 were also tested for proteinase activity. In these assays, no differences could be observed between these two proteinases, which both displayed activities similar to those of nsp4His. Taken together, these results confirmed the activity of the EAV nsp4His and MBP-nsp4 proteinases that had been purified from *E. coli*.

3.4. Nsp4 cleavage assay with synthetic peptide substrates

The *in vitro* cleavage assay described in the previous paragraph is less useful for quantitative biochemical analysis of nsp4

catalytic activity, since the quantification of the substrate is not straightforward. Consequently, an alternative assay based on the use of synthetic peptides as substrates was developed. Using this approach other 3C-(like) proteinases, like those of the human coronavirus 229E, the feline infectious peritonitis virus, and several picornaviruses (Cordingley et al., 1989; Hammerle et al., 1991; Hegyi et al., 2002; Jewell et al., 1992; Ziebuhr et al., 1997), have been successfully characterized.

A set of 16-mer peptides (Table 1), mimicking the P 9–P 7' positions of the six nsp4 cleavage sites downstream of nsp5 (Ziebuhr et al., 2000), was synthesized and the peptides were tested for their potential to be cleaved. In the initial experiment, 10 μ g of each peptide (final concentration approximately 100 μ M) was incubated at 25 °C with 37.5 μ g of either nsp4His or nsp4His-S120I (both at a concentration of 18 μ M), or without nsp4 proteinase, in a 100- μ l volume of a buffer containing 20 mM Tris pH 8.1, 150 mM NaCl, 1 mM EDTA. Samples were taken directly after addition of nsp4 ($t = 0$ h) or after 3- and 6-h incubations and were analyzed by reversed-phase HPLC. As an example, the results for peptide 222-36, representing the nsp7/8 cleavage site, are shown in Fig. 4. Following incubation with nsp4His, the main peak representing the uncleaved peptide was reduced and the peptide was apparently converted into two new products. Incubation with nsp4His-S120I or an assay without nsp4His did not yield these two new peaks, indicating that they were derived from nsp4His activity. When mass spectrometry was used to analyze the two newly formed products, it was found that the peaks with retention times of 8 and 9.7 min (Fig. 4) were of the same mass as the C-terminal and N-terminal fragments of the peptide, respectively, assuming cleavage at the Glu/Gly bond representing the established EAV nsp4 cleavage site in this substrate. All peptides could be cleaved under the selected conditions (approximately 25% hydrolysis after 6 h) without obvious differences between the peptides being detected (data not shown). The peptide representing the nsp7/8 cleavage site was selected to further optimize reaction conditions because of its slightly higher cleavage potential.

Although the purified EAV nsp4His proteinase showed catalytic activity on all peptides, its activity was very weak compared to that of other 3C-like proteinases in similar experiments (Cordingley et al., 1989; Hammerle et al., 1991; Hegyi et al., 2002; Jewell et al., 1992; Ziebuhr et al., 1997). Therefore, to investigate whether a differently purified nsp4 proteinase might perform better, the catalytic activity of nsp4His proteinase was compared to that of a partially purified MBP-nsp4 proteinase. The MBP-nsp4 protein was not further purified after the thrombin cleavage step, but instead the cleaved fusion protein sample was directly used in the peptide cleavage assay. A similar experimental set-up using HCV 229E 3CL_{pro} had shown that such a limited purification did not negatively affect the ability of the proteinase to cleave peptides (Ziebuhr et al., 1995). However, the enzymatic properties of the partially purified EAV MBP-nsp4 were not different from those of the (His)₆ tagged nsp4 proteinase (data not shown), suggesting that the low catalytic activity had a different cause. The possibility of inhibition of the proteinase by contaminants or instability of the enzyme after storage was investigated by an additional wash on the column

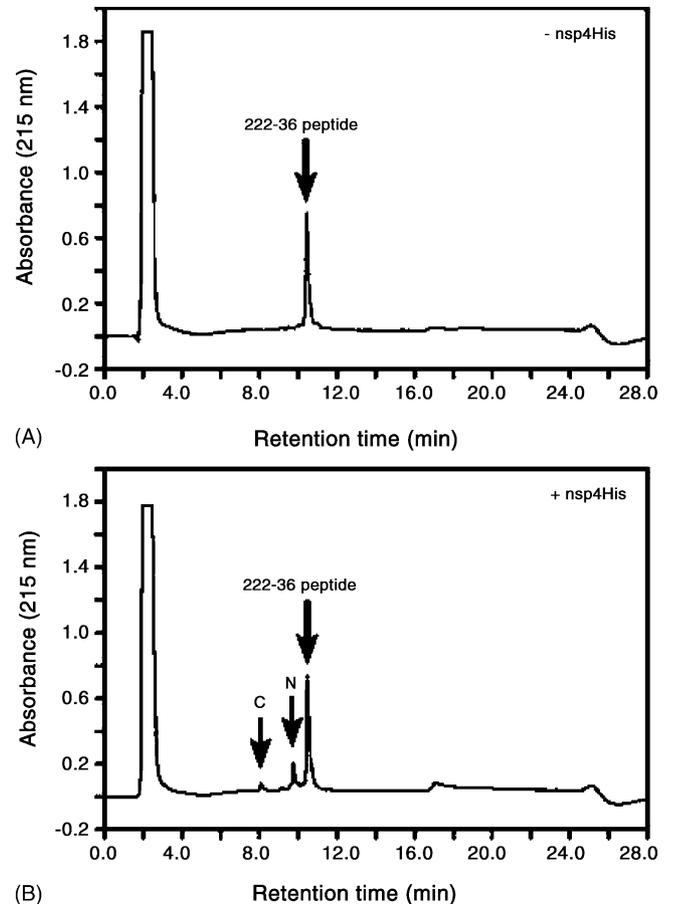


Fig. 4. EAV nsp4His activity in an *in vitro* cleavage assay using synthetic peptide substrates. One hundred micromolars of peptide 222-36 was incubated with 10 μ M nsp4His according to the protocol described in Section 2 and reaction products were analyzed by reversed phase HPLC. The results are shown for a reaction without proteinase (panel A) and a reaction to which nsp4His had been added (panel B). By using mass spectrometry, the peaks with retention times of 8 and 9.7 min in the HPLC profile of the sample incubated with nsp4His were identified as the C-terminal- and N-terminal fragments, respectively, of the 222-36 peptide substrate.

with 60% isopropanol (Franken et al., 2000) or by using the purified protein directly in the assay instead of after storage at 4 °C or –80 °C. However, the additional washing step did not increase catalytic activity and no differences were found between a freshly prepared sample and preparations stored at 4 °C for 3 days or stored at –80 °C for 2 weeks. This indicated that catalytic activity was not lost during storage, but was already low upon isolation of the enzyme, possibly due to the purification method and/or intrinsic properties of nsp4. Alternatively, the parameters of the *in vitro* cleavage reaction might have been suboptimal. To address the latter possibility, the catalytic activity of nsp4His was compared at different temperatures (20, 25, 30 and 37 °C) using the starting buffer composition (20 mM Tris pH 8.1, 150 mM NaCl, 1 mM EDTA). Although hydrolysis of the peptide turned out to be slightly faster at 37 °C, the differences in comparison to the other temperatures were marginal (data not shown).

In an attempt to further optimize the conditions of the cleavage assay, 20 μ g of peptide 222-36 (final concentration 200 μ M)

was incubated with 17.5 μg of nsp4His (final concentration 8.3 μM) in a volume of 100 μl at 37 °C in different buffers that varied systematically in pH, NaCl and glycerol concentration. The pH range tested included 6 (25 mM MES), 7 (25 mM MOPS), 8 (25 mM Tris), and 9 (25 mM glycine) and these pH values were combined with NaCl concentrations of 25, 500, and 1000 mM and glycerol concentrations of 10, 20, and 30%. After a 4-h incubation, the reaction was stopped by adding 100 μl of 10% trichloroacetic acid and samples were analyzed using reversed-phase HPLC. Since the peak with a retention time of 8 min, representing the C-terminal part of the peptide, had a very low absorbance at 215 nm, only the peak with a retention time of 9.7 min (Fig. 4) was quantified by integration. Samples could not be quantified when less than 3% of the substrate was cleaved. Although differences in catalytic activity were observed using the different buffers, no significant improvements were found compared to the starting reaction conditions in which 15% of the substrate was cleaved during a 4-h incubation at 37 °C. In the pH 6 buffer and at NaCl concentrations of 500 and 1000 mM, the catalytic activity of nsp4 was even below detection limit. No significant differences were found between reactions performed at pH 7, 8, or 9, but catalytic activity seemed slightly enhanced by increasing the concentration of glycerol (data not shown). Since high NaCl concentrations inhibited nsp4His activity, cleavage conditions were further optimized by titrating the NaCl concentration with 50-mM increments between 0 and 500 mM at a pH of 7.5 (25 mM MOPS) and a glycerol concentration of 10%. Although the catalytic activity was slightly higher at glycerol concentrations of 30%, a lower concentration was chosen for technical reasons. However, increasing the NaCl concentration above 25 mM only reduced the catalytic activity in the assay (data not shown). Finally, the effect of the presence of 10 mM DTT and 2.5 mM EDTA on catalytic activity was investigated, but addition of these compounds did not make a detectable difference (data not shown).

4. Discussion

This report describes two protocols for the expression and purification of recombinant EAV nsp4 and two approaches to assess the proteolytic activity of the purified enzyme *in vitro*. Both purification protocols resulted in the isolation of highly pure nsp4 proteinase, a conclusion supported by the subsequent use of the nsp4 moiety of MBP-nsp4 crystallization and structural studies (Barrette-Ng et al., 2002).

The results from the assays in which we used substrates synthesized in rabbit reticulocyte lysates showed that the nsp4 proteinase was able to process its cognate cleavage sites in the nsp6–8 and nsp7–8 substrates, indicating that an active enzyme had been purified. Although this was not a quantitative assay, the EAV nsp4 proteinase displayed kinetics that were comparable to those of other 3C-like proteinases in similar experiments (Grubman et al., 1995; Thole and Hull, 2002; Tibbles et al., 1996, 1999; Ziebuhr et al., 1995). The unexpected small reaction products visible in Fig. 3, at first suggested aberrant nsp4 activity or additional cleavages by other proteinases in the system, possibly following activation by nsp4. However, re-examination of

protein gels from several previous independent experiments, in which we used pp1a expression systems and EAV-infected cells to investigate nsp4 activity confirmed the existence of minor bands of approximately the same size as the unidentified bands in Fig. 3, making it more plausible that these bands were derived from genuine nsp4 activity. A more elaborate analysis of the presence of internal cleavage sites for nsp4 in nsp7 will be presented elsewhere (van Aken et al., manuscript in preparation).

To characterize nsp4 catalytic activity in more detail, assays using synthetic peptides as substrates were developed. For optimization of this cleavage assay, nsp4His was preferred over MBP-nsp4 because the purification of the latter was a more elaborate process. Although it was reported that the addition of six His residues strongly inhibited the enzymatic activity of the human coronavirus 229E 3CL^{PRO} proteinase (Ziebuhr et al., 1997), in both our assays the catalytic activity of nsp4His was very comparable to that of partially purified uncleaved or cleaved MBP-nsp4. Also, the *in vivo trans*-cleavage activity of nsp4His, as determined using the recombinant vaccinia virus/T7 expression system (data not shown), was comparable to that of untagged nsp4, suggesting that the presence of the (His)₆ tag did not affect nsp4 proteolytic activity.

Although the processing kinetics of *in vitro* synthesized substrates by nsp4His seemed comparable to that of, e.g. the distantly related human coronavirus 229E 3CL^{PRO} in an identical experimental set-up, this was not true for the synthetic peptide cleavage assays. Despite our efforts to improve the performance of the enzyme by testing a variety of reaction conditions, nsp4 was unable to cleave more than 15% of its substrate during a 4-h incubation, whereas studies using the human coronavirus 229E and SARS-coronavirus 3CL^{PRO} enzymes reported complete conversion of a comparable amount of substrate in less than 2 h, using a 10-fold lower enzyme concentration compared to that used in our nsp4 experiments (Shi et al., 2004; Ziebuhr et al., 1997). A possible explanation for this discrepancy is the lack of nsp2 in our assay, which was suggested to act as nsp4 cofactor in the polyprotein processing via the major pathway *in vivo* (Wassenaar et al., 1997). In the Flaviviridae family, the NS4A cofactor (Hepatitis C virus) and the NS2B cofactor (Dengue virus type 2) have been shown to enhance *in vitro* NS3 proteinase activity up to 100-fold (Steinkuhler et al., 1996; Yusof et al., 2000). Yet another explanation may be found in the observations made during the crystallographic studies of nsp4 of Barrette-Ng et al. (2002), who reported that three out of four copies of nsp4 in the unit cell had a collapsed oxyanion hole, which would render the proteinase practically inactive. If the majority of the purified nsp4His and MBP-nsp4 molecules would have had such a collapsed oxyanion hole, this might explain the low activity of both proteinases. Possibly this is a consequence of nsp4 expression in *E. coli*, since both forms of the proteinase (MBP-nsp4 and nsp4His) displayed low proteolytic activity. Expression in a different system (e.g. insect cells or yeast) might give better results. Also, we cannot rule out that the foreign amino acid residues present at either the N- or C-terminus of the recombinant nsp4 might have inhibited its proteolytic activity. In coronavirus main proteinases, the N-terminus was found to be of critical importance to the geometry of in particular the S1 subsite, suggesting

that changes (or extensions) in this part of the protein may adversely influence proteolytic activity (Anand et al., 2002). If this property is shared with the distantly related arterivirus main proteinase, this might explain the finding of the collapsed oxyanion hole (Barrette-Ng et al., 2002) in the majority of the molecules. Although it seemed that either only a small part of the purified nsp4 was proteolytically active or that the catalytic activity of the enzyme was low, the cleavage of cognate sites in both assays confirmed the specificity of the *E. coli*-derived nsp4 proteinase. Thus, the work described in this study may lay the groundwork for the more extensive biochemical characterization of the EAV main proteinase, including studies aiming to identify inhibitors of proteolytic activity.

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