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Expression and Characterization of Recombinant Hepatitis A Virus 3C Proteinase

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ABSTRACT: The 3C proteinase from the hepatitis A virus (HAV) was cloned into a multicopy expression vector in *Escherichia coli* under control of the *tac* promoter. The resulting plasmid construction produced 3C proteinase as a soluble and active enzyme constituting approximately 10% of total cellular proteins. The enzyme was purified to apparent homogeneity as judged by SDS gel electrophoresis and HPLC reversed-phase and FPLC ion-exchange chromatography. A colorimetric assay was developed, and synthetic peptides derived from the predicted cleavage sites of the HAV polyprotein were tested for proteolysis of the enzyme. The peptide representing the 2B/2C cleavage site was cleaved most efficiently with a K_m and k_{cat} of 2.1 ± 0.5 mM and 1.8 ± 0.1 s^{-1}, respectively. Site-directed mutagenesis was then used to identify the cysteine at position 172 as the active site nucleophile. Finally, the purified enzyme showed the expected endoproteinase activity on the P1 precursor protein generated by in vitro transcription/translation.

Hepatitis A virus is a member of the picornavirus family which appears to represent a distinct new genus (Jia et al., 1991). Picornaviridae, which include poliovirus and rhinovirus, possess a small (7-8-kb) positive strand RNA genome and a nonenveloped icosahedral capsid. The HAV[1] genome has been cloned and found to possess an organization characteristic of these viruses (Linemeyer et al., 1985; Najarian et al., 1985). Translation of this genome yields a polyprotein of approximately 251 kDa. Analysis of the picornaviral family suggests

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[1] Abbreviations: HIV, human immunodeficiency virus; HAV, hepatitis A virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

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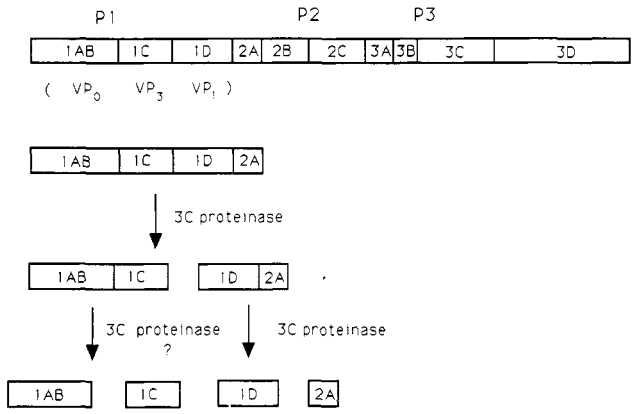
Scheme I: Genome Organization of HAV and Processing of Capsid Precursor (P1) (Not Drawn to Scale)



that the 3C region of the HAV genome encodes an endoproteinase responsible for processing the precursor into mature viral proteins (Scheme I). Precise locations of the processing sites in the noncapsid region of the HAV polyprotein are unknown, but they have been inferred by homology with poliovirus.

Most proteins in poliovirus are produced by 3C proteinase cleavage of the polyprotein between glutamine/glycine pairs. The complete determinants for substrate recognition and hydrolysis are unknown, but they probably include amino acid residues distal to the site of cleavage as well as the three-dimensional conformation of the polyprotein, since only 8 of 13 potential glutamine/glycine pairs are cleaved (Toyoda et al., 1986). This specificity for glutamine/glycine pairs is not strictly conserved among the picornaviruses (Krausslich & Wimmer, 1988); in particular, it is known that mature capsid proteins of HAV are produced by cleavages between a glutamine/methionine and a glutamine/valine pair (Linemeyer et al., 1985).

The enzymatic mechanism of the 3C proteinases has not been characterized in detail. Inhibition by thiol-specific reagents such as iodoacetamide, *N*-ethylmaleimide, and *p*-(chloromercuri)benzoate has implicated a cysteine residue at the active site (Korant, 1973; Pelham, 1978). A comparative study of the proteinases from several picornaviruses as well as from related plant potyviruses has revealed a region of significant homology in the carboxyl third of these proteins; in particular, a cysteine and a histidine appear to be conserved (Bazan & Fletterick, 1989). Furthermore, the subsequent site-specific mutagenesis of these residues in the poliovirus 3C proteinase rendered the proteinase inactive (Ivanoff et al., 1986). These observations support the conclusion that the picornaviral 3C proteinases are cysteine proteinases.

The expression and purification of proteinases from human immunodeficiency virus (HIV) (McKeever et al., 1989; Krausslich et al., 1989; Meek et al., 1989; Leob et al., 1989), poliovirus (Ivanoff et al., 1986; Nicklin et al., 1988; Pallai et al., 1989; Takahara et al., 1989), and rhinovirus (Libby et al., 1988; Knott et al., 1989) have led to a greater understanding of viral replication. Furthermore, the observation that many viruses encode proteinases for the posttranslational processing of virally encoded polyproteins has suggested that specific inhibitors of these enzymes might be effective antiviral compounds. In this paper, the overexpression in *Escherichia coli* and purification of active 3C proteinase from HAV as well as a peptide-based colorimetric assay are described. Site-directed mutagenesis and in vitro processing experiments are then described that confirm the type and specificity of the endoproteinase.

Materials and Methods

Molecular Biology. Restriction endonucleases and T4 DNA ligase, were obtained from New England Biolabs. Calf intestinal alkaline phosphatase came from Boehringer-Mannheim, and T4 polynucleotide kinase was from U.S. Biochemicals. Recombinant DNA techniques were performed according to Sambrook et al. (1989). DNA synthesis was as described (Warner et al., 1984), and DNA sequencing was by the dideoxy method of Sanger et al. (1977) using Sequenase version 2.0 from U.S. Biochemical Corp. (Cleveland, OH) with [α-^{32}P]dATP (800 Ci/mmol) from Amersham (Arlington Heights, IL). HAV sequences were derived from cDNA clones previously described (Najarian et al., 1985). The numbering of the HAV sequence is as in Najarian et al. (1985). Uracil-labeled single-stranded DNA templates were generated in strain RZ1032 using M13 subclones of HAV-3C. Site-directed mutagenesis was performed following the methods of Kunkel et al. (1987). DNA was transferred into *E. coli* strains using the one-step method of Chung et al. (1989).

Bacterial Strains and Plasmids. Max efficiency DH5αF'IQ competent cells (φ80d/*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 hsdR17*(r_K^-, m_K^+) *supE44* λ^- *thi-1 gyrA relA1/F' proAB*$^+$ *lacI*^{q}ZΔM15 zzf::Tn5[K_m^r]) were obtained from Bethesda Research Laboratories. Expression strain D1210-(*hsdS20, supE44, ara14, galK2, lacY1, proA2, rspL20, xyl5, mlt-1, recA13, mcrB, mcrA, mrr, lacI*^{q}*lacY*$^+$) was obtained from Dr. Robert Hallewell of Chiron Corporation. M13 propagation strain RZ1032 (Hfr KL16 PO/45 *lys A(61–62) thi-1 relA1 spoT* 1 *dut-1 ung-1*Zbd279::Tn10 (isogenic with BV313)) was obtained from Dr. Thomas Kunkel, NIEHS. Plasmid ptac5SOD containing the *tac* promoter was the generous gift of Dr. Robert Hallewell of Chiron Corporation.

Protein Analysis. N-Terminal sequence analysis was performed using an Applied Biosystems Model 470A gas-phase protein sequencer equipped with an on-line phenylthiohydantoin-amino acid analyzer at Chiron Corporation. Samples of purified C172A mutant proteinase were analyzed on a Jasco J500A spectropolarimeter at the University of California at San Francisco. Purified proteins were analyzed by reversed-phase, size-exclusion HPLC and ion-exchange FPLC in addition to SDS–PAGE. Reversed-phase columns (C-4, 4.6 mm × 25 cm, Vydac) were eluted with a linear 1%/min gradient (A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile). Size-exclusion columns (TSK-G2000SW, 7.5 mm × 25 cm, Supelco) were run under isocratic conditions (50 mM potassium phosphate, pH 7.0, 2.5 mM EDTA, 2 mM dithiothreitol). Ion-exchange chromatography columns (Mono S, 8-mL bed volume, Pharmacia) were eluted with a linear gradient of sodium chloride (0–1.0 M) in running buffer (50 mM Tris-HCl, pH 7.6, 2.5 mM EDTA, and 2 mM dithiothreitol). Protein concentration was measured by the Coomassie dye binding method (Bradford, 1976) as well as by quantitative amino acid analysis.

Synthesis and Purification of Peptides. Peptides were synthesized using an ABI Model 430A peptide synthesizer by solid-phase Fmoc methods (Atherton & Sheppard, 1989) on Rink resin (Rink, 1987) to generate amidated carboxy termini. In addition all amino termini were capped with acetic anhydride. After cleavage and deprotection with 95% TFA and appropriate scavengers (Atherton & Sheppard, 1989), the crude peptides were purified by HPLC, on a reversed-phase column (C-18, 2.2 × 25 cm, Vydac) using a 0–60% acetonitrile gradient (1%/min) containing 0.1% trifluoroacetic acid at a flow rate of 9.9 mL/min. The compositions and concentrations

Table I: Purification of HAV-3C Proteinase from 2 L of *E. coli* D1210 Harboring Plasmid pHAV-3CEX

purification step	protein (mg/mL)	total volume (mL)	total protein (mg)	sp act. [μmol/mg·hr)]	purification (x-fold)	yield
cleared lysate	1.22	190	231.8	0.20	N/A	100
DEAE eluant	0.58	170	98.5	0.32	1.6	67.9
CM-Sepharose concd pool	1.13	10.5	11.9	2.39	11.95	61.3

of stock solutions of these substrate peptides were determined by amino acid analysis. Amino acid compositions were obtained by using the picotag method of Waters Associates (Bidlingmeyer et al., 1984). Analyses were performed on samples hydrolyzed in the vapors of constant-boiling HCl (Pierce Chemical Co.) containing 1% (v/v) phenol for 22–24 h, in vacuo, at 110 °C prior to derivatization.

Assay of Proteinase Activity. All proteolysis reactions were conducted at 25 °C for 30 min in 94 mM sodium phosphate, 6 mM sodium citrate, 2 mM EDTA, 0.1 mM DTT, pH 7.6. Fragments from the proteolysis of the substrate peptides were separated by reversed-phase HPLC (C-18, 2.2 × 25 cm, Vydac; using a 0–60% acetonitrile gradient 1%/min) and sent for amino acid analysis to confirm the site of cleavage between the glutamine and the P_1' residues.

HAV-3C proteinase activity was monitored in a discontinuous fashion by following the release of amino groups during proteolysis from N-terminally acetylated substrate peptides using the reagent trinitrobenzenesulfonate (TNBS) (Billich & Winkler, 1990). Proteinase and substrate were incubated in reaction buffer at 25 °C. Four to ten 10-μL aliquots were removed from the reaction mixture at timed intervals, and peptide lysis was quenched with 50 μL of 0.25 M borate, 0.13 M NaOH. A total of 10 μL of freshly prepared 0.14 M TNBS in 0.25 M sodium borate solution was added to the quenched reaction mixture, and the mixture was incubated for 10 min at room temperature. The color was stabilized by adding 225 μL of 3 mM Na_2SO_3, 0.2 M KH_2PO_4. The concentration of free amine generated during peptide lysis was determined by measuring the absorbance at 405 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA) and ΔSoft software (BioMetallics, Princeton, NJ). The rate of cleavage was determined to be linear with respect to enzyme concentrations below 0.7 μM. All initial rate determinations were carried out at enzyme concentrations of 0.20–0.40 μM. The initial rates of cleavage for each peptide concentration, determined from time points measurements representing ≤30% of total substrate cleaved, were linear by regression analysis (KaleidaGraph; Abelbeck, Reading, PA). The k_{cat}/K_m values were calculated from nonlinear least-squares fit of initial rates as a function of substrate concentration (EnzymeKinetics; Trinity Software, Campton, NH). Estimates of amine released (absorbance at 405 nm) were calibrated with an independently synthesized carboxyl cleavage fragment that had not been acetylated (NH_2-S-F-S-NH_2) and had been quantified by amino acid analysis. Activity measurements during purification (Table I) were made using reversed-phase HPLC analysis at 520 nm of an alternate peptide substrate (T-P-L-S-T-Q-G-I-N-D-E-K-NH_2) previously labeled on the amino terminus with 4-(*N*,*N*-dimethylamino)azobenzene 4′-isothiocyanate (Chang, 1983).

In Vitro Translation of HAV P1 Precursor and Proteolysis by Purified HAV-3C. For expression of P1-2A, an *Asp*718 restriction site was added immediately upstream of the Met_1 codon of the HAV polyprotein by site-directed mutagenesis. The 3′ end of the P1-2A (1ABCD-2A) coding sequence was constructed by addition of a 33-bp oligonucleotide adaptor at the HAV *Avr*II site to extend the coding sequence to Gln_{856}. This oligonucleotide also incorporated a synthetic stop codon followed by a unique *Xho*I restriction site. For expression of HAV proteins in vitro an *Asp*718–*Xho*I fragment containing the P1-2A was inserted into pGEM-4Z-gb, an SP6 RNA polymerase promoter vector (pGEM-4Z, Promega) which was modified by addition of a synthetic 54-bp β-globin untranslated region at the *Eco*RI site and addition of an *Xho*I linker at the *Hin*dIII site.

Substrate markers and additional substrates for 3C proteinase were obtained by PCR subcloning. PCR adaptor-primers were synthesized for subcloning of VP_0 (1AB), VP_3 (1C), VP_1 (1D), VP_1-2A′ (1D-2A), and VP_0-VP_3 (1ABC). The adaptor portion contained a *Asp*718 site 5′ to the initiator methionine (Met_1 for VP_0 and VP_0-VP_3, Met_{246}, for VP_3). For expression of VP_1 and VP_1-2A, an artificial methionine codon was incorporated into the adaptor primer 5′ to Val_{492}. PCR was performed according to standard protocols, and the resulting DNA segments were inserted into pGEM-3Z-gb, aT7 polymerase promoter vector analogous to pGEM-4Z-gb.

Preparation of RNA for in vitro translation of P1-2A was performed using SP6 RNA polymerase (Promega) and linearized plasmid template according to the manufacturer's instructions. In vitro translation of P1-2A RNA was performed in nuclease-treated rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. [^{35}S]Methionine (>3000 Ci/mmol, Amersham) was used for labeling. Translation was performed at 30 °C for 1 h.

For processing studies, [^{35}S]methionine-labeled P1-2A or other substrates were prepared by in vitro translation, quick-frozen, and stored at −80 °C until use. Standard 75-μL translation reactions were used as the source of substrate. The frozen translation reactions were thawed and divided (35 μL each), and 2 μg of either inactive mutant C172A or active 3C proteinase was added. The processing reactions were incubated at 30 °C, and aliquots (16 μL) were taken at 3 and 6 h. Processing reactions were stopped by addition of SDS gel sample buffer and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

Results

Subcloning and Expression of 3C Proteinase. The coding sequence for HAV-3C protein was obtained from a HAV cDNA clone (Najarian et al., 1985). Synthetic oligonucleotides were added to the ends of a 616-bp *Xba*I–*Xmn*I fragment of HAV cDNA to generate a sequence encoding 219 amino acids with an artificial methionine initiation codon followed by Ser_{1520} and ending with Glu_{1736} followed by a translation stop codon (Figure 1). The coding sequence was flanked by unique *Asp*718 and *Sal*I restriction sites. The HAV-3C proteinase coding sequence used corresponded to that predicted by Diamond et al. (1986). This synthetic HAV-3C coding sequence was inserted into the *Asp*718–*Sal*I sites of the vector pGEM3Z (Promega) to generate pGEM3Z-3C^W.

Plasmid pGEM3Z-3C^W was digested with *Xba*I and *Sal*I (see Figure 1). The 657-bp insert containing 3C was purified by electrophoresis on a 1.5% agarose gel. An adaptor was generated by phosphorylating and annealing two synthetic oligonucleotides, which reconstructed the postulated amino terminus of the 3C proteinase, added an initiation codon, and inserted a translation enhancing minigene (Hallewell et al., 1985) upstream of the DNA encoding the 3C proteinase. The adaptor, 3C fragment, and gel purified *Nco*I–*Sal*I-digested plasmid ptac5SOD were ligated with T4 ligase overnight at 15 °C, the ligation mixture was transformed into *Max efficiency* DH5αF′IQ *E. coli* (BRL) and colonies were selected

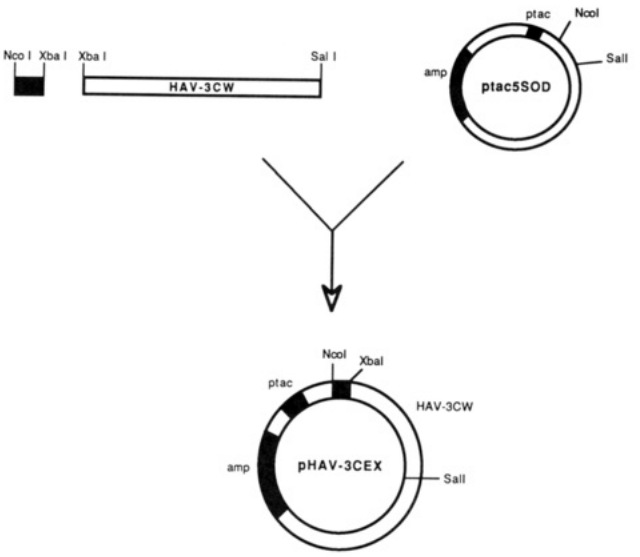


FIGURE 1: Overexpression of HAV-3C proteinase. The *Xba*I–*Sal*I fragment was excised from the pGEM3Z and fused via the synthetic adaptor shown below, which contains the translation enhancing minigene, into the ptac5SOD vector for expression. The resulting dicistronic construct is under the control of the *tac* promoter and is induced by the addition of IPTG (Materials and Methods).

5′ CATGGCTACAGAGGAATTATAATATGTCAACT
CGATGTCTCCTTAATATTATACAGTTGAGATC 5′

on ampicillin (150 μg/mL) LB agar plates. Recombinant clones were identified by digestion of DNA minipreps with restriction endonucleases yielding plasmid pHAV-3CEX. The newly generated *Nco*I–*Sal*I fragment of pHAV-CEX was excised and subcloned into M13 vectors and sequenced in its entirety by the Sanger method (Sanger et al., 1977).

Growth and Purification. Transformation of pHAV-3CEX into *E. coli* D1210 resulted in a bacterial strain that expressed the 3C proteinase under the control of the *tac* promoter. Fresh overnight cultures of *E. coli* D1210 harboring plasmid pHAV-3CEX were inoculated into 2 × YT (1.6% bactopeptone, 1.0% yeast extract, 0.5% NaCl) containing 150 μg/mL ampicillin and incubated at 30 °C on a rotary shaker at 325 rpm. When the OD_{650} reached 0.4, isopropyl β-D-thiogalactopyranoside was added to the culture to a final concentration of 2 mM. After 6 h of induction, the bacteria were harvested by centrifugation (10 min at 2000*g*) and resuspended in 50 mM Tris-HCl (pH 8.5) and 2.5 mM EDTA, 2 mM DTT (100 mL/L of culture). Lysozyme was added to a final concentration of 80 μg/mL, and the mixture was incubated on ice for 30 min. Lysis was achieved by freezing and thawing the mixture three times. Bacterial debris were cleared by sedimentation (9000*g* for 60 min), and the supernatant was collected. The supernatant was then gently stirred with an equal volume of DEAE-Sephadex A-25 (Pharmacia) preequilibrated in the lysis buffer. The slurry was centrifuged at 2000*g* for 5 min, and the supernatant was applied to a CM-Sepharose column (Pharmacia, 100 mL of bed volume/L of culture) previously equilibrated in lysis buffer. After sample application, the column was washed with three column volumes of 50 mM Tris, pH 8.5, 0.25 mM EDTA, 2 mM DTT and then eluted with a 0–1.0 M NaCl linear gradient in the same buffer. Column fractions were analyzed by SDS–PAGE (Laemmli, 1970), and those fractions containing 3C proteinase as judged by the appearance of a protein of 24 kDa were pooled, concentrated, and dialyzed against 50 mM potassium phosphate (pH 7.5), 2 mM DTT, 0.25 mM EDTA using

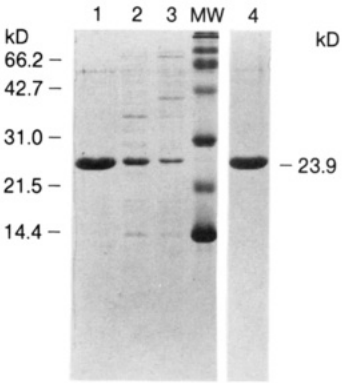


FIGURE 2: Coomassie-stained SDS–PAGE analysis of HAV-3C proteinase purification: Lane 1, CM-Sepharose pooled fractions; lane 2, DEAE eluant; lane 3, cleared lysate; lane 4, C172A HAV-3C inactive mutant. All lanes contain 2.5 μg of protein.

Amicon centriprep molecular filtration units and stored at −80 °C (see Table I). The purified protein was stable for several months with no appreciable loss of activity.

When induced with IPTG, intracellular levels of 3C proteinase reached approximately 10% of total cellular protein as shown in Figure 2. A simple two-step purification (Table I) resulted in 3C proteinase >95% pure as judged by SDS-PAGE (Figure 2). HPLC reversed-phase and size-exclusion chromatography as well as FPLC ion-exchange chromatography confirmed the purity of the proteinase (data not shown). Over 12 mg of active proteinase was obtained from 2 L of cells. Edman sequencing revealed that the N-terminal methionine was quantitatively removed and that the amino acid sequence S-T-L-E-I-A-G-L-V-R-K-N matched that encoded by the DNA sequence.

Peptide Assay Development and Kinetic Analysis. The cleavage site peptides were derived from putative cleavage sites on the HAV polyprotein (Krausslich & Wimmer, 1988). Proteolysis of all eight cleavage site peptides, followed by reversed-phase HPLC, suggested that the peptide from the 2B/2C junction was the most rapidly cleaved (Jewell et al., manuscript in preparation). A minimal peptide from this sequence (ac-E-L-R-T-Q-S-F-S-NH_2) was synthesized and used for the development of the TNBS colorimetric assay. Initial rate measurements (consuming <20% of the substrate peptide) yielded a K_m and k_{cat} of 2.1 ± 0.5 mM and 1.8 ± 0.1 s^{-1}, respectively, assuming enzyme preparations were 100% active (Figure 4).

Site-Directed Mutagenesis of HAV-3C. In order to confirm the role of cysteine 172 as the active site nucleophile of the HAV-3C proteinase, the mutant C172A was generated by site-directed mutagenesis. The synthetic oligonucleotide

5′ CCTGGAATGGCTGGTGCCCTAGTG 3′

was used to replace the putative active site cysteine at position 172 in the amino acid sequence with an alanine while an *Apa*I site was simultaneously removed so that screening could be performed by restriction digestion. Mutant clones were subsequently verified by Sanger dideoxy sequencing prior to subcloning into plasmid ptac5SOD for expression. The insert was subcloned back into ptac5SOD after sequence confirmation and expressed in *E. coli* D1210. The mutant C172A was grown and purified exactly as described for the wild-type enzyme and by all previous criteria (SDS–PAGE and reversed-phase and size-exclusion HPLC as well as ion-exchange FPLC) appeared equally pure. The mutant proteinase showed no proteolytic activity.

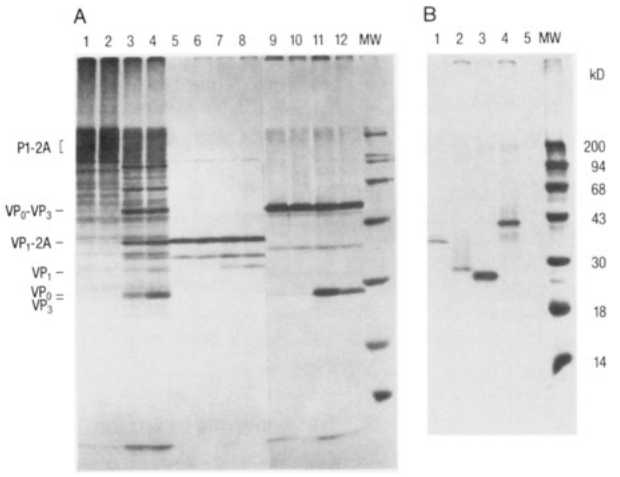


FIGURE 3: Processing of P1-2A, VP_0-VP_3, and VP_1-2A by HAV-3C proteinase. Panel A: [^{35}S]methionine-labeled P1-2A capsid precursor was incubated either with inactive mutant proteinase C172A for 3 h (lane 1) or 6 h (lane 2) or with active 3C proteinase for 3 h (lane 3) or 6 h (lane 4). [^{35}S]Methionine-labeled VP_1-2A (lanes 5–8) and VP_0-VP_3 (lanes 9–12) also were incubated with inactive mutant proteinase for 3 h (lanes 5 and 9) and 6 h (lanes 6 and 10) or with active 3C proteinase for 3 h (lanes 7 and 11) or 6 h (lanes 8 and 12). The positions of P1-2A (93.6 kDa), VP_0-VP_3 (55.0 kDa), VP_1-2A (38.6 kDa), VP_1 (33.2 kDa), VP_0 (27.8 kDa), and VP_3 (27.3 kDa) are indicated. Other unique cleavage products of P1-2A with apparent mobilities of 88 kDa and 66 kDa may represent P1 and VP_3-VP_1-2A, respectively. All lanes shown in panel A are from a single gel, but a shorter exposure of lanes 9–12 + MW was used to allow better comparison with lanes 1–8. Sizes of the markers are as indicated in panel B. Panel B: In vitro translation of synthetic RNAs encoding VP_1 (lane 1), VP_0 (lane 2), VP_3 (lane 3), and VP_1-2A (lane 4) are shown. Products of a translation reaction minus RNA are shown in lane 5.

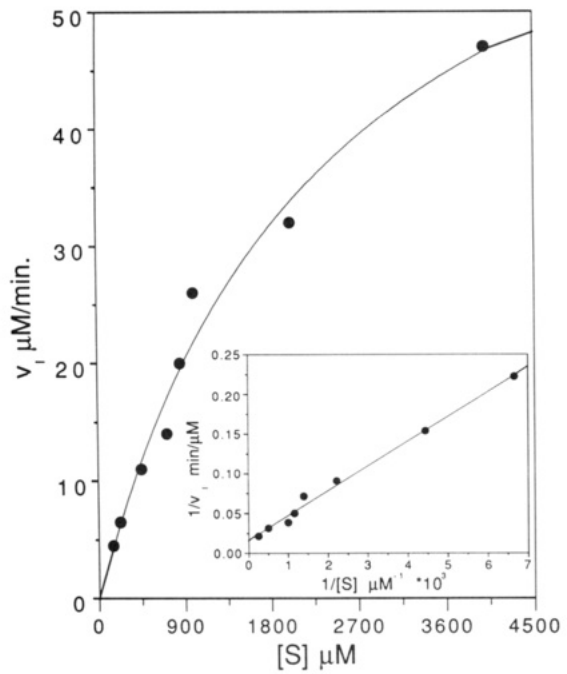


FIGURE 4: Kinetics of peptide hydrolysis by HAV-3C proteinase. Michaelis–Menten fit of initial velocity dependence on substrate concentration. Hydrolysis was measured at 25 °C in 80 μL of 94 mM potassium phosphate, 6 mM sodium citrate, pH 7.5, using the TNBS assay as described under Materials and Methods. Errors on indicated values are ±10%. Insert: Lineweaver–Burke representation of the kinetic data. K_m = 2.1 ± 0.5 mM and k_{cat} = 1.8 ± 0.1 s^{-1}.

Proteolysis of Capsid Precursors Generated by in Vitro Translation. [^{35}S]Methionine-labeled capsid precursors (P1-2A, VP_0-VP_3, and VP_1-2A) were prepared by in vitro translation and subsequently incubated with either wild-type HAV-3C proteinase or the inactive mutant C172A. Analysis of the products by SDS–PAGE is shown in Figure 3 (panel A). To identify the positions of the capsid proteins and partially cleaved precursors, constructs encoding the various products were generated via PCR and used to prepare synthetic RNA for in vitro translation (Figure 3, panel B). No processing of the capsid precursors was observed with the inactive mutant C172A. Incubation of P1-2A precursor with wild-type 3C proteinase generated partially cleaved precursors (VP_0-VP_3, VP_1-2A) plus capsid proteins VP_0, VP_3, and VP_1. Cleavage of the precursors VP_0-VP_3 to generate VP_0 + VP_3 and VP_1-2A to generate VP_1 was also observed, although at slower rate than P1-2A. Proteolytically processed VP_0 and VP_3 were somewhat difficult to resolve (Figure 3, panel A). VP_1-2A and VP_1 expressed from synthetic RNAs appear to run slightly higher than the corresponding fragments cleaved from the P1-2A precursor. The reason for this difference in mobility is not clear, but it may result from the presence of the additional methionine residue.

Discussion

This paper reports the overexpression and development of a high throughput assay for the HAV-3C proteinase, a member of the picornaviral 3C proteinase family and thus permits a comparison of specificity and catalytic properties with other members of this novel class of cysteine proteinases. To date, only limited studies have been performed; most information has been inferred by analogy with the thoroughly studied poliovirus 3C and rhinovirus 3C enzymes (Kay & Dunn, 1990; Weidner & Dunn, 1991). The overexpression of multimilligram quantities of HAV-3C proteinase per liter of culture also makes practical structural investigation by X-ray crystallography, CD spectroscopy, NMR spectroscopy, etc.

The high level of proteinase expression made purification a relatively straightforward problem. Following the lead of Cordingly et al. (1989), the estimated isoelectric point of the protein (pI = 9.2) was used to develop a simple purification scheme. By conducting anion-exchange chromatography at pH 8.5, the vast majority of *E. coli* proteins in addition to the nucleic acids are bound to the resin and thus eliminated from the lysate. The subsequent cation-exchange chromatography (also at pH 8.5) allows effective resolution of the 3C proteinase from the remaining basic proteins, primarily lysozyme added during lysis.

The natural cleavage site peptides were tested for sensitivity to 3C proteolysis in order to generate a convenient HAV-3C proteinase assay (Dunn et al., manuscript in preparation). Monitoring of proteolysis reactions by reversed-phase HPLC suggested that, unlike other picornaviral 3C proteinases studied to date (Kay & Dunn, 1990), the HAV 2B/2C sequence was the most sensitive to proteolysis by its cognate 3C proteinase (Dunn et al., manuscript in preparation). With the use of this information, a truncated form of the 2B/2C sequence (acetylated at the amino terminus and amidated at the carboxy terminus) was synthesized and used as the basis for a rapid, discontinuous, colorimetric assay. This assay, based on the reaction of TNBS with the amino groups generated during proteolysis, is very similar to the method developed independently by Billich and Winkler (1991).

By homology with the previously mutagenized poliovirus 3C (Ivanoff et al., 1986), the cysteine at position 172 was predicted to be the active site nucleophile. Using site-directed mutagenesis, this was converted to alanine in an effort to verify the prediction and generate an inactive proteinase as a control for the precursor processing studies. In both the peptide-based

assays and the precursor-processing studies, the C172A mutant showed absolutely no proteolytic activity. In order to assure that this was not merely due to an improperly folded enzyme, 1D-NMR spectra were taken of the HAV-3C proteinase and the inactive mutant. Spectra were essentially identical (data not shown), suggesting that the absence of activity was not due to gross misfolding of the enzyme.

The results of the precursor processing experiment are comparable to those seen previously with the poliovirus system (Nicklin et al., 1988). As with poliovirus the most extensive cleavage of the P1 precursor occurred between VP_3 (1C) and VP_1 (1D) yielding VP_0-VP_3 (1ABC) and VP_1-2A (1D-2A) fragments of 55.0 kDa and 38.6 kDa, respectively (Figure 3). However cleavage of these fragments into VP_0, VP_3, and VP_1 also was observed (Figure 3). This is consistent with studies on poliovirus 3C proteinase generated in *E. coli* reported by Nicklin et al. (1988). These studies and those of Ypma-Wong et al. (1988) suggest that the 3CD precursor is required for complete and rapid processing of the poliovirus P1 precursor to the individual capsid proteins. In context, complete and efficient cleavage of capsid precursors by 3C proteinase has been observed in EMCV and FMDV (Parks et al., 1989; Ryan et al., 1989).

Recent studies by Jia et al. (1991) and Gauss-Muller et al. (1991) using in vitro translation and recombinant fusion protein approaches to examine HAV-3C proteinase processing of polyprotein precursors conclude that the enzyme works only in cis. The data presented in this report suggest that the HAV-3C proteinase is capable of acting in trans to site-specifically cleave the P1 precursor as well. Further studies are in progress to further assess the role of 3C versus 3CD in the effective processing of the HAV polyprotein.

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Registry No. Ac-E-L-R-T-Q-S-F-S-NH_2, 139408-94-9; cysteine, 52-90-4; 3C proteinase, 37353-41-6.

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