Expression and Purification of Recombinant 3C Proteinase of Coxsackievirus B3

Kinji Miyashita,† Mikiko Kusumi, Ryutaro Utsumi,∗ Tohru Komano,** and Nobukatsu Satoh

Central Research Laboratories, Maruishi Pharmaceutical Co., Ltd., 2-2-18 Imazumakura, Tsurumi-ku, Osaka 538, Japan
∗Laboratory of Biochemistry, Department of Agricultural Chemistry, Kinki University, 3327-204 Nakamachi, Nara 631, Japan
**Laboratory of Biochemistry, Department of Agricultural Chemistry, Kyoto University, Sakyoku-ku, Kyoto-606, Japan

Received September 30, 1991

We have cloned various lengths of coxsackievirus B3 cDNA encompassing the region encoding the 3C proteinase, which is essential to the viral replication cycle. Such viral cDNAs were fused in frame to the 5′terminal portion of the lacZ gene carried on the vector pUC118 to express mature 3C proteinase in Escherichia coli. In the E. coli cells containing pCXB108 or pCXB117, constructed for this study, a large amount of 23-kDa protein was synthesized in the presence of IPTG. This protein was purified and was shown to be intact 3C proteinase. These data suggest that 3C proteinase, expressed as a part of a fusion protein, was active in E. coli and released itself from the precursor fusion protein by autocatalytic cleavage.

Coxsackieviruses, members of the enterovirus subgroup of the picornavirus family, have a single-stranded RNA genome of about 7400 nucleotides1−5 with positive polarity. In common with all picornaviruses, coxsackieviruses translate their RNA genome into a large polypeptide, which is processed through a series of proteolytic cleavages to produce both capsid and nonstructural proteins6−8 as shown in Fig. 1.

For enteroviruses and rhinoviruses, members of another subgroup of the picornavirus family, there are at least two known proteinases responsible for these proteolytic cleavages encoded in the genome. The 2A proteinase has been shown to be responsible for the initial cleavage of the polypeptide between the P1 and P2 regions.9,10 This event is thought to take place while the peptide is still nascent on a ribosome. All remaining cleavages are accomplished by the 3C proteinase11 or one of its precursors,1,2,13 except for the processing of VP0 to VP4 and VP2. The cleavage of VP0 occurs during encapsidation of the RNA and the mechanism of this event remains obscure.14,15 Thus the 3C proteinase plays a major role in the viral replication cycle, and this proteinase is thought to be a suitable target for antiviral chemotherapy. To design specific inhibitors of viral proteinases, it is desirable to purify and characterize the proteinase. For these purposes recombinant DNA technology is useful because of the small quantities of 3C protein produced in infected cell cultures; the 3C proteinases from poliovirus,16−19 human rhinovirus (HRV),20−22 and hepatitis A virus (HAV)23 have been expressed in Escherichia coli.

Here we report cloning and expression of coxsackievirus B3 (CVB3) cDNA segments encompassing the 3C coding region in E. coli. Each plasmid was constructed to express 3C proteinase by autocatalytic cleavage from the precursor protein in E. coli. Two of the 6 constructed plasmids induced the synthesis of fairly large amounts of the 23-kDa protein corresponding to 3C proteinase. This 23-kDa protein was purified and was shown to be active 3C proteinase.

Materials and Methods

Bacterial strains, media, enzymes, and plasmids. The E. coli strain JM109 (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ (lac−proAB), [F−, traD36, proAB, lacPZΔM15]) was used throughout this work. LB medium consists of 1% bacterotrypton, 0.5% yeast extract, and 1% NaCl. Restriction endonucleases, the Klenow fragment of DNA polymerase I, T4 DNA ligase, and pUC vectors were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) and Toyobo Co., Ltd. (Osaka, Japan).

Virus purification. The CVB3 strain Nancy obtained from Dr. A. Iigaki, Shimane Prefectural Institute of Public Health, was twice plaque-purified and propagated in LLC-MK2 cells grown in roller bottles. Confluent cell monolayers were infected with CVB3 at a multiplicity of infection of 10 to 20 PFU per cell. Infected cells were incubated in Eagle minimum essential medium without serum until the cytopathic effect was complete. Cells were disrupted by freezing and thawing. After debris was removed by centrifugation, polyethylene glycol 6000 and NaCl were added to 7% and 0.5 M, respectively, and the mixture was stirred at 4°C overnight. The precipitated virus was recovered by centrifugation and suspended in phosphate-buffered saline (PBS). NP-40 (detergent) was then added to 1% and the virus particles were purified by centrifugation on a 15 to 30%
surrogate density gradient. Fractions containing virus, identified by measuring the optical density at 260 nm, were harvested and precipitated by centrifugation. The pellet was suspended in RSB (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂) and banded in a CsCl density gradient (5 to 40%) after addition of NP-40. The virus was collected, diluted with RSB, and finally purified by centrifugation on a discontinuous sucrose gradient (30 and 60%). Purified virus was stored at −40°C.

Construction of plasmids. Purified virus was digested with proteinase K (500 µg/ml) for 5 min at 37°C and SDS was then added to 1%. After 5 min of incubation at 37°C the solution was extracted twice with phenol and the aqueous phase was reextracted with chloroform. RNA was recovered by ethanol precipitation, suspended in water, and used as a template for cDNA synthesis.

Preparation of cDNA was done by the protocol of a cDNA synthesis kit (Amersham) using oligo(dT) as a primer. This CVB3 cDNA was digested with HindIII and from the resulting 4.6-kb fragment was inserted at the HindIII site of pUC118 from which the Smal–HindIII 17-bp fragment had been deleted. A recombinant plasmid in which the orientation of the viral genome was the same with that of the lacZ gene of the vector was selected and designated pCXB102. This plasmid was treated with SphI and T4 DNA ligase to generate pCXB103.

To clone the 3’ end of the viral genome, viral cDNA was treated with SclI and the resulting 1-kb fragment (one end of this fragment was a blunt end) was inserted into pUC19 that had been digested with Smal and SclI. The resulting plasmid was digested with HindIII and the resulting 0.7-kb fragment was then ligated with pCXB103 digested with HindIII to generate pCXB104.

To construct pCXB105, pCXB104 was digested with DraiI and Xhol and the resulting 2.6-kb fragment was inserted into pUC118 cleaved with Smal and Xhol. Then pCXB105 was treated with EcoRI, EcoRII, the Kleenso fragment, and T4 DNA ligase to generate pCXB108. From this plasmid, a HindIII 1.3-kb fragment was deleted to generate pCXB117.

Nucleotide sequence analysis. Double-stranded plasmid DNA was denatured in 0.2 M NaOH at room temperature for 5 min and recovered by ethanol precipitation. The nucleotide sequence was identified by the dideoxy chain termination method using a Thd DNA sequencing kit (Toyobo) and [γ-32P]ATP.

Expression of viral cDNA in E. coli and identification. An E. coli strain harboring one of the expression plasmids was grown overnight in LB medium containing 50 µg/ml ampicillin. Of this culture, 0.2 ml was used to inoculate 10 ml of LB medium containing ampicillin and incubated at 37°C until OD₆₀₀ = 0.3, at which time IPTG was added to 1 mM. After 3 hr of incubation, 1 ml of cells was centrifuged and the cell pellet was suspended in 0.5 ml of buffer and boiled for 5 min. SDS-PAGE was done using a precast gel (TEFB systems) and proteins were stained with Coomassie brilliant blue R-250.

Purification of 3C proteinase. An overnight culture of E. coli strain harboring pCXB117 was diluted 1:50 in 300 ml of LB medium containing 50 µg/ml ampicillin and grown at 37°C until OD₆₀₀ = 0.3, when IPTG was added to 1 mM. After 3 hr of incubation, cells were harvested by centrifugation and the cell pellet was stored at −80°C or suspended in 5 ml of buffer A (50 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 5 mM dithiothreitol) containing 1 mg/ml lysozyme.

All subsequent purification steps were done at 4°C. The cells were broken by ultrasonication; 5 x 30 sec bursts. The suspension was centrifuged for 15 min at 12,000 rpm. Ammonium sulfate was then added to the supernatant to 15% saturation and centrifuged for 10 min at 10,000 rpm. The supernatant was then put on a Buryl–Toyopearl 650M column (1.5 x 19 cm), precolumnated with buffer B (50 mM Tris–HCl, pH 8.0, 1 mM dithiothreitol) containing 15% saturated ammonium sulfate. Bound proteins were eluted with a linear gradient of ammonium sulfate (15% saturation to 0%) in buffer B. Fractions containing 3C proteinase, eluting between 12.5 and 9% saturated ammonium sulfate, were detected by SDS-PAGE and pooled. To concentrate the protein, ammonium sulfate was added (350 mg/ml) and the mixture was left overnight. The precipitate was collected by centrifugation for 15 min at 12,000 rpm, suspended with buffer C (40 mM Tris–HCl, pH 8.4, 1 mM dithiothreitol), and dialyzed against the same buffer. Insoluble material was removed by centrifugation for 10 min at 10,000 rpm, and the supernatant was put on a DEAE-Celulose A-500 (1.5 x 17 cm), precolumnated with buffer C. The proteinase was eluted with a linear gradient of NaCl (0 to 0.1 M) in buffer C. Protein elution was monitored at 280 nm and peak fractions, eluting around 40 mM NaCl, were pooled, concentrated by ultrafiltration, and finally purified by gel filtration chromatography using a Cellulofine GCL-500-m column (1.5 x 72 cm) in buffer D (50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol). Peak fractions were pooled, concentrated, and stored at −80°C after addition of glycerol to a final concentration of 15%.

Measurement of protein concentration. Protein was measured by the Bio-Rad protein assay. Bovine plasma γ-globulin was used as the standard protein.

Amino acid sequence analysis. The N-terminal sequence was analyzed by automated Edman degradation using a solid phase sequencer (MiliGen/ Biosearch model 6600).

Peptide synthesis. The peptide was synthesized with an Applied Biosystems 430A peptide synthesizer on phenylacetic acid methyl polystyrene resin by NMP-HOBt Boc cycle. The crude peptide was purified by reverse-phase HPLC with a 30 to 50% gradient of acetonitrile containing 0.1% trifluoroacetic acid.

Peptide cleavage assays. The peptide (1 mg/ml) was incubated with 3C proteinase (40 µg/ml) for 14 hr at 30°C in a final volume of 40 µl buffer E (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol). The reaction was stopped by boiling for 5 min and the mixture was centrifuged for 5 min to remove insoluble material before analysis by reverse-phase HPLC on a Bondapack C₁₈ column (3.9 x 150 mm). Cleavage products were eluted with a 30 min linear gradient of 0 to 50% acetonitrile in 0.1% trifluoroacetic acid.

Results

Cloning of coxsackievirus B3 cDNA encoding 3C proteinase

Plasmid constructions were done referring to the CVB3 restriction map derived from the nucleotide sequence reported by Lindberg et al.,¹³ and constructed plasmids were checked by restriction enzyme analysis. Six plasmids were thus constructed to fuse viral genes in frame to the 5’ end of the lacZ gene carried on pUC118. Viral cDNAs cloned in these recombinant plasmids are shown in Fig. 2.

To confirm that the 3C proteinase was encoded, the DNA sequence of the viral cDNA in pCXB117 was analyzed by the dideoxy chain termination method. As shown in Fig. 3, pCXB117 contains 20 nucleotides encoding the C-terminal part of 3B, the entire 3C coding region (549

![Fig. 2. Cloning of CVB3 3C Proteinase Gene.](image-url)

Gene organization of CBV31–21 is shown at the top of the figure. VPG and poly(A) are explained in the legend of Fig. 1. Viral cDNAs cloned in recombinant plasmids are shown under the gene structure. The region encoding 3C proteinase is shaded. Other viral sequences are unshaded. Numbers represent nucleotide positions on the CVB3 genome. These cDNAs were fused in frame to the 5’ end of the lacZ gene, carried on the vector, pUC118. Procedures for plasmid construction are described in Materials and Methods.
plasmids, pCXB102, pCXB103, pCXB104, pCXB105, pCXB108, and pCXB117, were constructed as mentioned above. These plasmids were used to transform the E. coli strain JM109, and the transformed strain was grown and treated with the inducer IPTG as described in Materials and Methods. Cells were harvested 3 hr after addition of IPTG and proteins were analyzed by SDS-PAGE.

Synthesis of a 23-kDa protein was induced by IPTG in the cells containing pCXB108 or pCXB117 (Fig. 4, lanes 3 and 5) while it was not observed in non-induced cells (Fig. 4, lanes 2 and 4) or induced cells carrying pUC118 alone (Fig. 4, lane 1). An extra 54-kDa protein corresponding to 3D polymerase was also observed in induced cells containing pCXB108 (Fig. 4, lane 3), which contains the entire 3C and 3D coding region (Fig. 2). In contrast, no induction of 3C gene expression by IPTG was observed in the cells containing pCXB102, pCXB103, pCXB104, or pCXB105 (data not shown).

These results suggest that mature 3C proteinase was produced by autocatalytic cleavage from the precursor protein in E. coli cells carrying pCXB108 or pCXB117 and that the 3D protein was also produced in pCXB108-containing cells. The densitometer scanning of the SDS-PAGE gel showed that the amount of the 23-kDa protein produced in pCXB117-containing cells reached 5–10% of total cellular protein. To examine whether this 23-kDa protein is the 3C proteinase, we purified this protein, sequenced its N-terminal amino acids, and measured its activity to cleave a synthetic peptide substrate.

**Purification of 3C proteinase**

Since the 23-kDa protein exists mainly in the soluble fraction, after disruption of the cells, it was purified from the supernatant of the bacterial cell lysate as described in Materials and Methods. Purified samples were analyzed by SDS-PAGE (Fig. 5).

Butyl-Toyopearl 650M chromatography resulted in the removal of a large amount of bacterial cell proteins (Fig. 5, lane 2 compared with lane 1). Most of the residual impurities were removed by the next step, DEAE-Cellulose
Expression and Purification of CVB3 3C Proteinase

Fig. 5. Purification of Recombinant 3C Proteinase.

The 3C proteinase was purified from the soluble extracts (lane 1) of E. coli cells carrying pCXB117 with Butyl-Toyopearl 650M (lane 2), DEAE-Cellulofine A-500 (lane 3), and Cellulofine GCL-300-m (lane 4). Samples were analyzed by 0.1% SDS-16% PAGE and proteins were stained with Coomassie brilliant blue. Molecular size markers are indicated in lane M.

Fig. 6. Reverse-phase HPLC Analysis of Synthetic Peptide Substrate Cleaved by Purified 3C Proteinase.

The substrate peptide was incubated with (B) or without (A) 3C proteinase for 14h at 30°C. Samples were analyzed on a µBondasphere C18 column in a linear gradient from 0 to 50% acetonitrile in 0.1% trifluoroacetic acid.

A-500 chromatography (Fig. 5 Lane 3) and the 23-kDa protein was further purified by Cellulofine GCL-300-m chromatography (Fig. 5, lane 4).

The N-terminal sequence of this purified protein was analyzed up to residue 10 by automated Edman degradation. The sequence obtained, GPAFFEFVAM, corresponds to the N-terminal amino acid sequence predicted from the cDNA sequence (Fig. 3), indicating that the purified protein is the 3C proteinase of CVB3.

Enzymatic activity of purified 3C proteinase against a synthetic peptide

Recently, two groups demonstrated that several peptides whose sequences corresponded to viral polyprotein cleavage sites were cleaved by the 3C proteinase in vitro with varied efficiencies using 3C proteinases of poliovirus25 and HRV14,26 respectively. In both cases, the peptides representing the 2C/3A cleavage site were effective substrates. Therefore, we synthesized a 16-amino-acid peptide with a sequence TTLAEALFQGPPVVYREI corresponding to the 2C/3A cleavage site of CVB3 as a substrate.

This peptide has a retention time of 31—32 min on reverse-phase HPLC (Fig. 6A, peak 1). When the peptide was incubated with purified 3C proteinase, the peak 1 diminished and two new peaks appeared at 24—25 min (Fig. 6B, peak 2) and 29—30 min (Fig. 6B, peak 3). These new peaks did not arise when the peptide was incubated with buffer alone (Fig. 6A). The product peaks were isolated and their amino acids sequenced, which showed that peak 2 was derived from the peptide GPPVVYREI and peak 3 was derived from the peptide TTLAEALFQ. These results indicate that the substrate peptide was cleaved by the recombinant 3C proteinase at the Gln-Gly bond which is the predicted 2C/3A cleavage site.

Discussion

Picornaviral 3C proteinase is essential in the viral life cycle and is produced from its precursor(s) by autocatalytic cleavage in infected cells. In previous reports, mature 3C proteinases of poliovirus16,17,19 HRV,20,22 and HAV23 have been shown to be produced when expressed as a part of precursor protein in E. coli. To express the 3C proteinase of CVB3 in E. coli, we constructed 6 recombinant plasmids in which viral sequences containing 3C coding region were fused to the 5' terminal part of the lacZ gene carried on the vector pUC118. However, neither the mature 3C proteinase nor its precursors accumulated in the cells containing pCXB102, pCXB103, pCXB104, or pCXB105. Viral cDNAs cloned in these plasmids might be too large to be expressed efficiently. Alternatively, the precursor proteins expressed from these plasmids might be unstable in E. coli. The other 2 plasmids, pCXB108 and pCXB117, directed synthesis of large amounts of a 23-kDa protein. Its activity to cleave a synthetic peptide substrate at the Gln-Gly bond and its N-terminal amino acid sequence indicate that this protein is active 3C proteinase and that the 3B/3C cleavage site was correctly processed in E. coli. The processing of the 3C/3D cleavage site in E. coli is suggested by the finding that additional 54-kDa protein corresponding to 3D polymerase was also produced in the cells containing pCXB108, which has the entire 3C and 3D coding region. The 23-kDa protein is slightly larger than the one calculated from the amino acid sequence (20,3-kDa) but similar results were obtained with poliovirus 3C proteinase.17,19 In these experiments, mature 3C proteinase migrated to a position of 24-kDa upon SDS-PAGE although its calculated molecular mass is 20-kDa. The 23-kDa protein, expressed in this study, is therefore considered to be a mature 3C proteinase, released by autocleavage. It is unlikely that these specific cleavages were done by bacterial proteinase(s) because previous reports23,27,28 showed that the processing of 3C precursors in E. coli was abolished by mutagenesis of the active site residue of 3C and we have also obtained similar results (data not shown).

The DNA sequence and predicted amino acid sequence
of pCXB117 (Fig. 3) show that the primary translation product induced in pCXB117-containing cells consists of 6 amino acids derived from β-galactosidase, the C-terminal 7 amino acids of 3B, the whole 3C sequence (183 amino acids), the N-terminal 66 amino acids of 3D, and an extra 16 amino acids derived from the vector sequence. (A Leu residue derived from fusion of the 5' end of viral cDNA and the vector sequence is identical with the corresponding amino acid of 3B peptide.) It is known that autocleavage of the 3C/3D site is inefficient. Polypeptide 3CD, the precursor to 3C and 3D, is stable in infected cells and previous reports showed that significant amounts of the precursors, in which the 3C/3D site remained uncleaved, accumulated in E. coli. In this study, neither the primary precursor protein (31-kDa) nor the intermediate precursor (29.5-kDa), in which the 3C/3D site remained uncleaved, accumulated in infected cells (Fig. 4, lane 5). These results are inconsistent with the previous findings. The reason for this inconsistency is not clear. Cordingley et al. reported, however, that most of the 3C/3D site of the HRV-14 3C precursor was cleaved in E. coli. Autoprocessing of this site may be affected by several factors, such as the viral strain, promoter used for expression, and solubility of the recombinant proteins. In addition, accumulation of a recombinant protein is strongly affected by its stability. In our expression system, the intermediate precursor protein might be somewhat less stable than the mature 3C protein.

In summary, we have expressed and purified active CVB3 3C proteinase. The availability of large quantities of purified 3C proteinase and its peptide substrate should facilitate the characterization of this viral-specific enzyme and the development of antiviral drugs that specifically inhibit the processing of viral polyprotein. Using various synthetic peptides, an attempt to elucidate the substrate specificity of this enzyme is now in progress.

Acknowledgments. We thank Asao Itagaki for providing CVB3 and Masanobu Agoh for helpful discussion and technical advice concerning virus propagation and purification of viral RNA.

References