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Endopeptidase activity characterization of *E. coli*-derived infectious bursal disease virus protein 4 tubules

Gary Ro-Lin Chang[1], Min-Ying Wang[1,4], Jiahn-Haur Liao[2], Yu-Ping Hsiao[3] and Su-Yuan Lai[3,4]

[1]Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan, [2]Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan and [3]Department of Food Science and Technology, Central Taiwan University of Science and Technology, 666 Buzi Road, Pei-tun District, Taichung, Taiwan

[4]To whom correspondence should be addressed.
E-mail address: sylai@ctust.edu.tw

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Viral protein 4 (VP4) is a serine protease that catalyzes the hydrolysis of polyprotein pVP2-VP4-VP3 of infectious bursal disease virus. In this report, the recombinant VP4 with a His-tag and three mutants (VP4-S652A, VP4-K692A and VP4-S652A.K692A) were expressed in *Escherichia coli*. Soluble VP4 was purified using immobilized metal-ion affinity chromatography or sucrose density gradient following with gel-filtration chromatography. The purified VP4 has a tubular structure with 25–30 nm in width and ~300 nm in length, as observed by transmission electron microscope. A similar tubular structure was also found for these three mutants. The endopeptidase activity of these VP4 tubules was characterized by fluorescence resonance energy transfer using a synthetic fluorogenic oligopeptide as a substrate. The results show that the tubule-like VP4 is a functional enzyme with K_m of 43 ± 2 μM and k_{cat} of 0.04 ± 0.01 min^{-1}; however, k_{cat} of three mutants were significantly reduced. This is the first report to demonstrate that VP4 protein expressed in *E. coli* can self-assemble into functional tubule-like particles and its activity can be completely inhibited by 1 mM of Ni^{+2} ions.

Keywords: endopeptidase activity/immobilized metal-ion affinity chromatography (IMAC)/infectious bursal disease virus/nickel ions/Ser/Lys dyad/tubule-like particles

Introduction

Serine proteases cleave the peptide bond by nucleophilic attack of the serine hydroxyl group with the assistance of one or more other residues. Classical serine proteases have a catalytic triad containing the hydroxyl group of the nucleophile Ser, the imidazole ring of His, which serves as a general acid/base, and the carboxylate group of an Asp, which assists to orient the imidazole ring. Proteases such as trypsin, chymotrypsin, Factor XI and elastase belong to this group (Dodson and Wlodawer, 1998; Botos and Wlodawer, 2007). The other type of serine protease, which is relatively rare, contains a Ser/Lys catalytic dyad in the active site, where Ser is the nucleophile that attacks the carbonyl atom of the scissile bond with its hydroxyl group, and Lys serves as a general base to accept the proton (Botos *et al.*, 2005). Proteases that utilize this mechanism are categorized into the evolutionary clans SE, SF, SJ and SK in the MEROPS peptidase database (Rawlings *et al.*, 2006). VP4 proteases of birnaviruses (Feldman *et al.*, 2006) and some bacterial proteases, including Lon protease (Botos *et al.*, 2004), type I signal peptidase (Paetzel *et al.*, 2002), LexA (Luo *et al.*, 2001), signal peptide peptidase (SppA) (Paetzel and Strynadka, 1999) and UmuD (Peat *et al.*, 1996) belong to this group.

Birnaviruses are non-enveloped viruses of ~65 nm, and are characterized by their two double-stranded RNA segments (A and B) (Dobos *et al.*, 1979). The well-characterized members of the birnaviruses include the blotched snakehead virus (BSNV) (Feldman *et al.*, 2006), the Tellina virus 1 (TV-1) (Chung and Paetzel, 2011), the infectious pancreatic necrosis virus (IPNV) (Lee *et al.*, 2007), Drosophila X virus (Chung *et al.*, 1996) and the infectious bursal disease virus (IBDV) (Hudson *et al.*, 1986). IBDV is the causative agent of a highly contagious immunosuppressive disease among young chickens (Kibenge *et al.*, 1988; Nagarajan and Kibenge, 1997). Its genomic segment B encodes a 90 kDa VP1 protein, which is the RNA polymerase (Morgan *et al.*, 1988). Segment A encodes a nonstructural protein (VP5) (Mundt *et al.*, 1997) and a 115-kDa polyprotein which can be proteolytically processed to yield pVP2, VP3 and VP4 proteins (Hudson *et al.*, 1986). The viral protease VP4 releases pVP2, VP3 and itself from the polyprotein (Lejal *et al.*, 2000). The pVP2 subsequently undergoes a second proteolytic cascade to generate mature VP2 capsid protein [amino acid (aa) 1–441] and four small peptides (aa 442–487, aa 488–494, aa 495–501, aa 502–512) (Da Costa *et al.*, 2002). The substrate cleavage motif for VP4 was defined as (Thr/Ala)-X-Ala↓Ala-(Ser-Gly) (Lejal *et al.*, 2000).

The structural information on VP4 proteins from birnaviruses has been elucidated recently. The first structure of VP4 solved was from a truncated form of BSNV without a substrate bound in the active site (Feldman *et al.*, 2006). The following solved structure was IPNV and it revealed an intermolecular (trans) acyl-enzyme complex (Lee *et al.*, 2007). TV-1 VP4 with C-terminus binding to its own active site forming an intramolecular (cis) acyl-enzyme complex has also been shown (Chung and Paetzel, 2011). VP4 protein of IBDV had been described to be associated with a type II tubule of 25 nm in IBDV-infected cells (Granzow *et al.*, 1997), characterized to use a Ser652/Lys692 catalytic dyad in the active site with both cis and trans activities (Lejal *et al.*, 2000). So far, the formations of tubules as well as the

enzymatic properties of VP4 tubules have not been documented.

In this work, recombinant VP4 protein of IBDV was over-expressed in *Escherichia coli*, purified to homogeneity and its protease activity was characterized using a synthetic fluorogenic oligopeptide. Our results show that the highly expressed VP4 can self-assemble into a tubular structure and the tubule can function as an endopeptidase *in vitro*. The effect of point-mutagenesis (S652 or/and K692) on the tubule formation and endopeptidase activity were also evaluated. To further characterize the VP4 tubules, effect of pH, temperature and some cations on the endopeptidase activity of VP4 tubules were also determined. Taking together, the methodologies shown here provide a strategy to obtain a large amount of thermostable VP4 tubules with endopeptidase activity for structural analysis and for the investigation of anti-IBDV drugs.

Materials and methods

Bacterial strains and anti-VP4 antibodies

E. coli strain TOP10F' (Invitrogen, NY, USA) was used for gene cloning and *E. coli* strain BL21(DE3)pLys (Stratagene, La Jolla, CA) was used for the expression of recombinant proteins. Rabbit anti-VP4 polyclonal antibodies were produced by immunizing rabbits with Ni-NTA agarose (QIAGEN, Valencia, CA)-purified VP4 protein derived from *E. coli* BL21(DE3)pLys strain (LTK BioLaboratories, Tauyuan, Taiwan).

Construction of a recombinant plasmid expressing IBDV VP4 protein

Plasmid pB4-IBD-A containing the cDNA coding for IBDV polyprotein (GenBank ID: AF109154) was constructed previously (Lee *et al.*, 2004). The DNA sequence corresponding to amino acid residues 513–755 of polyprotein was amplified by polymerase chain reaction (PCR) using plasmid pB4-IBD-A as a template and the primers VP4–513-F (5′-CTAGCTAGCATGGCTGACAAGGGGTACGAG-3′) and VP4-755-R (5′-CCGCTCGAGTCAAGCCATGGCAAGGTG GTA-3′). (The sites of *Nhe*I and *Xho*I were underlined.) The PCR product was introduced into the *Nhe*I and *Xho*I sites of vector pET28a (Novagen) to form the recombinant plasmid pET28a-VP4. The recombinant plasmid was transformed into competent *E. coli* TOP10F' for selection. Plasmid DNA was isolated, analyzed by restriction enzyme digestion and sequenced to confirm the correct coding sequence. Finally, the plasmid was transformed into competent *E. coli* BL21(DE3)pLys for VP4 expression.

Cloning and expression of VP4-S652A, VP4-K692A and VP4-S652A.K692A mutants

VP4 gene in plasmid pET28a, designated as pET28a-VP4, was used as a template. PCR primer pair, VP4 S652A-F (5′-GGAAACGCTGGCAACCTAGCCAT-3′), VP4 S652A-R (5′-GTTGCCAGCGTTTCCCACAATAG-3′) and primer pair VP4 K692A-F (5′-AAGCAACGCGCTCGCCACTGCACA-3′), VP4 K692A-R (5′-TGGCGAGCGCGGTGCTTCTAA AGC-3′) were used with the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). PCR reaction was performed as follow: starting at 94°C for 3 min, template denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min, extension at 72°C for 5 min. After 24 cycles of denaturation, annealing and extension, another 10 min was allowed to ensure complete polymerization. The reaction mixture was then treated with *Dpn I* to digest the template, followed by PCR clean up kit (Qiagen) to purify PCR product and finally the PCR-generated mutant plasmids were transformed into *E. coli* TOP10F' (Invitrogen). The resulting plasmids, pET28a-VP4-S652A and pET28a-VP4-K692A, containing mutant genes of VP4 were isolated and analyzed by restriction enzyme digestion and then sequenced to confirm the correct sequence. Plasmid pET28a-VP4-S652A.K692A was generated using pET28a-VP4-S652A as a template and a primer pair of VP4 K692A-F and VP4 K692A-R for PCR reaction as the procedures mentioned above. Following the verification of the recombinant plasmids by DNA sequencing, the plasmids were transformed into *E. coli* BL21 (DE3)pLys for protein expression.

Expression of VP4 and mutant proteins

A fresh overnight culture of *E. coli* BL21 (DE3)pLys carrying pET28a-VP4 or other mutant genes was used to inoculate 300 ml of fresh Luria-Bertani media supplemented with 50 μg/ml kanamycin and cells were grown to OD_{600} of 0.4–0.6 at 37°C. Protein expression was induced by adding isopropyl-β-thiogalactopyranoside (IPTG) at four different concentrations (0.25, 0.5, 0.75 and 1 mM) for 4 h at 37°C. To determine an optimal induction time, following induction, cells were harvested every hour and pelleted at 13 800 × *g* for 5 min. The pellets were mixed with sample buffer (25% glycerol, 2% sodium dodecyl sulphate, SDS, 60 mM Tris–HCl, 0.1% bromophenol blue, 5% β-mercaptoethanol) for protein identification by SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis.

Purification of VP4 and mutant proteins

In this study, proteins were purified by a two-step procedure. Firstly, the pellets of 100 ml induced *E. coli* culture were resuspended thoroughly in 1 ml of buffer A [20 mM Tris (pH 8.8), 100 mM NaCl, 1 mM dithiothreitol (DTT), 10% glycerol] and disrupted with sonication for 10 min. After centrifugation at 13 000 × *g* at 4°C for 30 min, the ice-cold cell-free extract was added with 1 ml of Ni-NTA resin (Qiagen, Valencia, CA). The batch binding of VP4 to resin was performed at 4°C for 1 h, and then the VP4-bound resin was loaded onto a column. To remove more impurities from the resin, the column was washed using a 10-fold resin volume of a wash buffer (buffer A supplemented with 20 mM imidazole). VP4 was then eluted by an elution buffer (buffer A supplemented with 250 mM imidazole). The collected fractions were resolved by SDS-PAGE and stained with Coomassie blue.

Secondly, the fraction containing VP4 was subjected to further purification by gel filtration. A Sepharose CL-4B column (1.6 × 36 cm) was packed and equilibrated with buffer A before sample injection. The column was mounted onto an automatic ÄKTApurifier FPLC system (GE) and eluted with buffer A at a flow rate of 0.5 ml/min. Fractions containing VP4 proteins were collected and resolved by SDS-PAGE to analyze their purity. The elution volumes for Blue dextran 2000 (2000 kDa) and trypsin (28 kDa), respectively, were also determined for this column.

VP4 Protein purification by sucrose density gradient and gel filtration

Five milliliters of cell-free extract containing protein was loaded onto a 20/30/60% sucrose density gradient and followed with ultracentrifugation at 35 000 rpm at 4°C for 4.5 h using Beckman Type 70 Ti rotor. Fractions of 1 ml were collected from the top and analyzed by SDS-PAGE. Sucrose densities of VP4-containing fractions were determined as previously described (Ho *et al.*, 2010). Concentrated protein samples containing VP4 (2 ml) were applied onto a Sepharose CL-4B gel filtration column (1.6 × 40 cm), and eluted using buffer I (20 mM Tris, pH 8.0, 100 mM NaCl and 1 mM DTT). Fractions monitored at 280 nm were collected and analyzed by SDS-PAGE. The molecular weight of VP4 tubule was estimated using the high-molecular-weight and low-molecular-weight gel filtration calibration kits (GE Healthcare, Uppsala, Sweden).

Western blotting analysis and protein quantification

Western blotting analysis was performed by separating the samples on a 12.5% SDS-PAGE gel, blotted onto a PVDF membrane, then reacted with an anti-VP4 polyclonal antibody. After washing, the membrane was color-developed in a 10-ml alkaline phosphatase buffer containing 66 μl of nitrobluetetrazolium and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate stock solution. The intensities of VP4 proteins in the stained membranes were quantified using 1D image analysis software version 3.6 (Kodak, NY, USA).

Electron microscopy

Negative-stain electron microscopy was used to examine the morphology of *E. coli*-derived VP4 tubules. The method for a direct observation through negative staining was performed as described previously (Lee *et al.*, 2004). Briefly, a 5-μl droplet of VP4 samples was placed on the surface of a 300-mesh grid covered with Formvar/carbon membranes, and incubated for 2 min. Following decantation, the grids were floated on a 2% uranyl acetate aqueous solution to achieve contrast. After decantation and drying, the grids were observed through a transmission electron microscope (JEOL JEM 1400, Tokyo, Japan) with a voltage acceleration of 120 kV.

VP4 protease activity assay

VP4 protease activity assay was performed by fluorescence resonance energy transfer (FRET) as described in Ekici *et al.* (2009) with modifications. The synthetic oligopeptide, substrate for VP4 protease, was synthesized using the standard Fmoc protocol (Merrifield, 1986). The peptide was designed as KGKARAASEG with the conjugations of Dabcyl (4-{[4-(dimethylamino)phenyl]azo}benzoic acid; a fluorescent quencher) at the N-terminal lysine and Edans (5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid; a fluorescent donor) at the glutamate residue. The molecular weight of this peptide is 1475.5 Daltons and a stock solution of 0.5 mg/ml in dimethyl sulfoxide was prepared and frozen at −20°C. Substrates of various concentrations were diluted from the stock solution using buffer A before use.

Protease activity assays were performed at 37°C in a 96-well black flat plate at a total volume of 100 μl containing VP4 and the peptide substrate in buffer A. The reaction was initiated by the addition of VP4 protein and the emitted fluorescence was monitored at the wavelength of 496 nm (excitation at 335 nm) every 2 min for 1–4 h on an Infinite M200 Pro microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Effect of pH and cation on VP4 activity

The effect of pH on VP4 protease activity was performed in a buffer (20 mM Tris, 100 mM NaCl and 1 mM DTT) at different pH values. The relative activity was defined as the ratio of the endopeptidase activity at a specific pH to that at pH 8.8. The effect of a divalent cation on the activity of VP4 was achieved by adding $MgCl_2$ (Mg^{2+}), $CaCl_2$ (Ca^{2+}), $MnCl_2$ (Mn^{2+}) or $NiSO_4$ (Ni^{2+}) to a final concentration of 10 mM in buffer A. After the addition of VP4 (3.45 μM), the fluorescence was monitored as described above. The relative activity was defined as the ratio of the endopeptidase activity at the buffer containing a specific divalent cation to that of buffer A.

Effect of temperature on VP4 tubule stability

To study the effect of temperature on the stability of the VP4 tubules, the enzymes were incubated at different temperatures for 30 min in buffer A. After heat treatment, the enzyme solutions were cooled quickly. Endopeptidase activities were determined before and after heat treatments as described above. The remained percentages of activities after the heat treatments were calculated.

Steady-state kinetic constants determination

Initial velocities were measured at 37°C using a 100-μl reaction solution containing 3.45 μM VP4 and varying concentrations of fluorogenic peptide (2–40 μM) in buffer A. The reaction was initiated by the addition of VP4 protein and monitored at 496 nm as described above. The amount of hydrolyzed peptide was measured by determining the maximum fluorescence generated per 0.5 μg (3.4 μM) peptide after complete digestion by 0.1 μg of trypsin. To determine the kinetic constants, the initial velocity data were fitted to Michaelis-Menten equation: $V_0 = V_{max}[S]/(K_m + [S])$, where [S] is the substrate concentration; V_0 is the initial velocity; V_{max} is the maximum velocity; and K_m is the Michaelis constant for the substrate. The k_{cat} value was calculated from V_{max} and the enzyme concentration [E] using the equation $k_{cat} = V_{max}/[E]$.

Results

Expression of VP4 and mutants in E. coli

The recombinant plasmid, pET28a-VP4, carrying the VP4 gene fused with the coding sequence of an extra 24 amino acids (MGSSHHHHHHSSGLVPRGSHMASM) at the N-terminus was constructed for VP4 expression. VP4 protein with the 6× histidine-affinity tag and the linker region was highly expressed in the *E. coli* strain BL21(DE3)pLys (Fig. 1A, lane 2). The expressed VP4 protein has a molecular mass, as revealed by SDS-PAGE, similar to the calculated molecular mass for this construct (~29.094 kDa). Quantitative analysis showed that the expression level of VP4 after 4 h of IPTG induction (0.75 mM) was ~28% of the total bacterial proteins (Supplemental Table SI). The expression

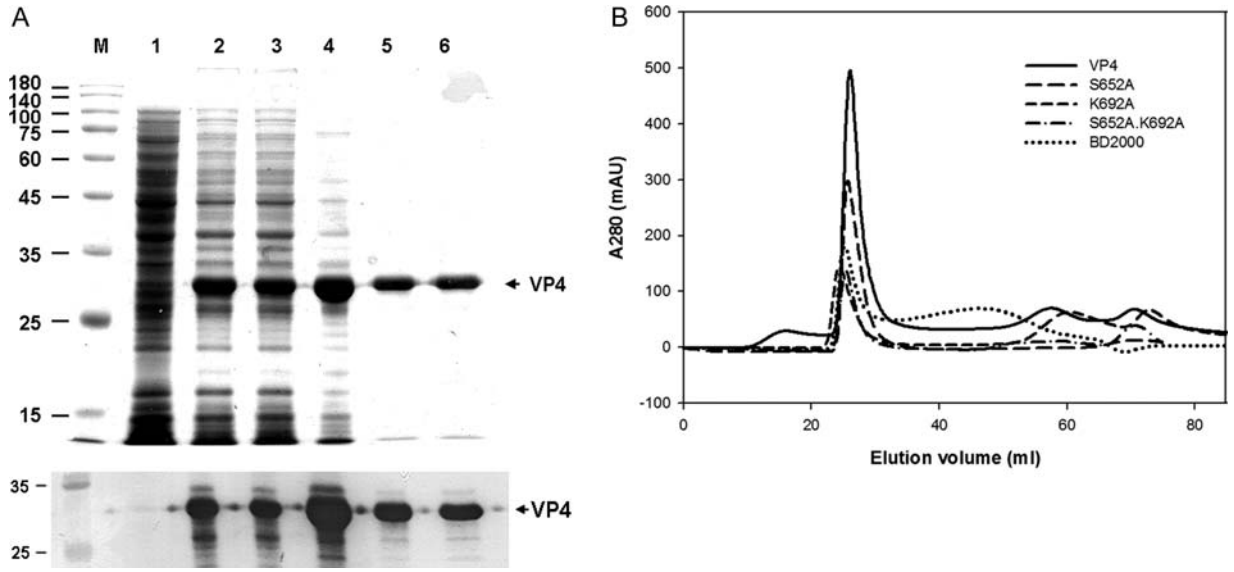

Fig. 1. Expression and purification of IBDV VP4 through IMAC and gel-filtration. (**A**) SDS-PAGE (up) and western blotting analysis (bottom) of VP4 protein expression and purification. The induced *E. coli* pellet was resuspended in buffer A and disrupted by sonication and followed with centrifugation to remove cell debris. Soluble VP4 in supernatant was purified by IMAC and CL-4B gel filtration. Lane M, prestained protein markers; lane 1, uninduced cell lysate; lane 2, IPTG-induced cell lysate; lane 3, soluble proteins after centrifugation; lane 4, an eluted fraction from IMAC; lanes 5 and 6, two consecutive fractions from the peak shown in Fig. 1B. (**B**) Elution profiles of VP4 and its mutants from a Sepharose CL-4B gel filtration column. The flow rate of mobile phase (buffer A) was 0.5 ml/min. Blue dextran 2000 with a molecular weight about 2000 kDa (BD2000) was loaded as a control.

profiles of three VP4 mutants (VP4-S652A, VP4-K692A and VP4-S652K692A) were similar to that of the wild-type VP4 (data not shown).

Purification of VP4 and mutants

Purification of VP4 was achieved using an IMAC column and following with a Sepharose CL-4B gel filtration column. The result showed that VP4 has a molecular weight of or >2000 kDa (Fig. 1B). Because the theoretical molecular mass of monomeric VP4 is about 29 kDa, the results suggested that VP4 formed multimeric complexes. Three VP4 mutants (VP4-S652A, VP4-K692A and VP4-S652A.K692A) were also purified by this two-step chromatography with a similar result. As shown in Fig. 1B, these three mutants also formed a multimeric complex.

To purify the high-molecular-weight VP4, we also used a 20/30/60% sucrose density gradient for VP4 preparation from the cell-free extract. After 4.5 h ultracentrifugation, two fractions of sucrose densities of 1.20–1.21 g/cm^3 were collected (Supplemental Figs. S1A and B, lane 5) and subjected to gel filtration for further purification. From the elution profile of VP4, the molecular weight of multimeric complex VP4 was also >2000 kDa when blue dextran 2000 (2000 kDa) and chymotrypsinogen A (25 kDa) were loaded as molecular mass controls (Supplemental Fig. S1C). This result suggested that the formation of VP4 multimeric complex was not due to IMAC purification. Therefore, IMAC combined with gel-filtration was employed for the purification of various VP4 multimeric complexes. In summary, ~2 mg of VP4 with a purity of ~97% was obtained from a batch of 100 ml induced *E. coli* culture, and the recovery of VP4 was about 16% (Supplemental Table SI).

Morphological analysis by electron microscopy

VP4 purified by the combination of IMAC or ultracentrifugation and gel filtration steps was then observed under an electron microscope (EM). As shown in Fig. 2, the EM analysis revealed that VP4 formed tubular structures. The tubules are 25 nm in width with cross-striations, and the distance of two adjacent cross-striations is ~5 nm. Although the morphology of the tubule is very similar to that of the type II tubule found in IBDV-infected chicken embryo fibroblast (CEF) cells (Granzow *et al.*, 1997), this is the first time to show that VP4 alone can form a tubular structure. The tubular structure was also found for all VP4 mutants (VP4-S652A, VP4-K692A and VP4-S652A.K692A) as shown in Figs 2B–D, suggesting that S652 and K692 are not involved in VP4 tubule formation.

An earlier study reported that intraplasmic and intranuclear type II tubules of ~25 nm in diameter containing VP4 were found abundantly in IBDV-infected CEF cells by immunoelectron microscopy using an anti-VP4 monoclonal antibody (Granzow *et al.*, 1997). Here, our results showed that the overexpression of VP4 itself in *E. coli* can trigger the self-assembly of VP4 into a structure similar to the type II tubules. Therefore, we strongly suggest that the type II tubules found in IBDV-infected cells contain VP4 only. Among these expressed VP4 proteases from aquatic birnaviruses, none has been confirmed to form a tubule-like structure. Although the presence of type II tubules in IBDV-infected cells was speculated to inactivate excess protease to prevent lethal damage to the virus or to the cells (Tacken *et al.*, 2003), it is interesting to determine the endopeptidase activity of the expressed VP4 tubules to confirm whether the tubular protease complex is totally inactive or not.

Endopeptidase activity of VP4

To examine the endopeptidase activity of the expressed VP4 tubule, the fluorogenic peptide K(Dabcyl)GKARAASE (Edans)G was synthesized based on the known *in vivo* cleavage sites of VP4 (Table I). The optimal pH for the endopeptidase activity of VP4 was then characterized. As shown in

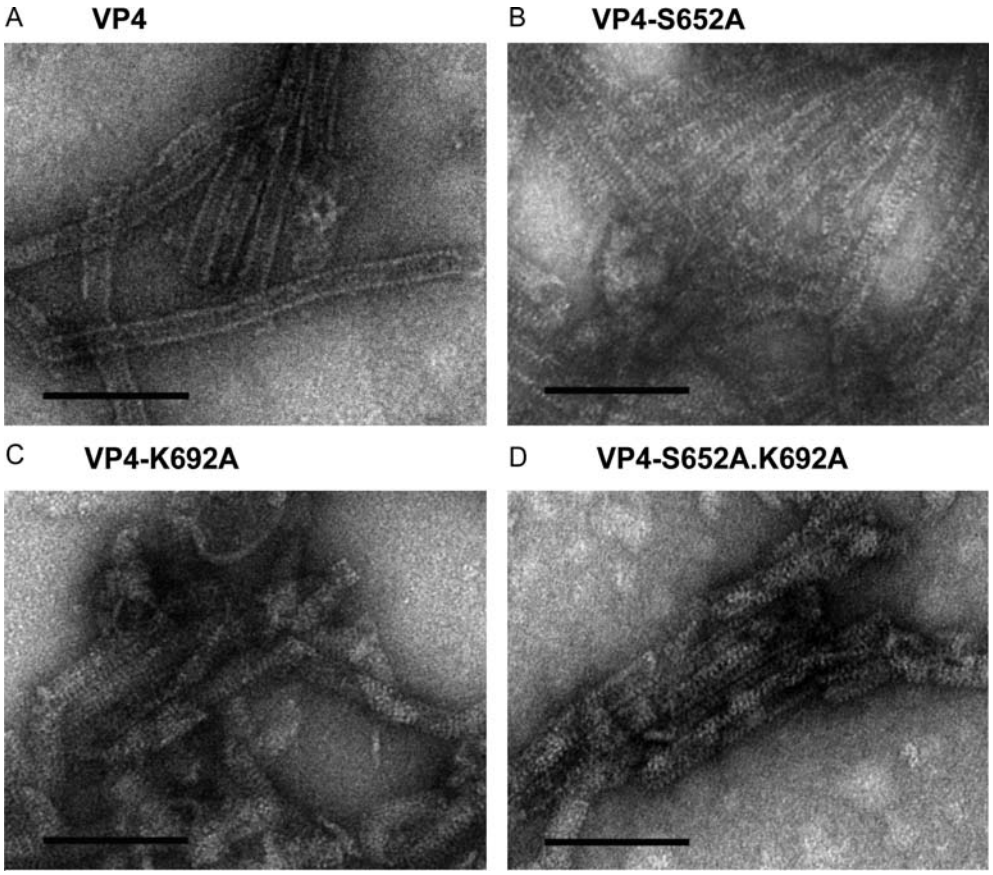

Fig. 2. Transmission electron micrograph of VP4-related tubules. The wild-type VP4 (**A**) and its respective mutants (**B**: VP4-S652A; **C**: VP4-K692A and **D**: VP4-S652A.K692A) were first purified by IMAC and Sepharose CL-4B gel filtration and then, subjected to EM (JEOL JEM 1400, Tokyo, Japan) observation. Uranyl acetate (2%) was used for staining. Bar: 100 nm.

Table I. *In vivo* cleavage sites of IBDV VP4

IBDV cleavage site	Peptide sequence
VP2-pVP2 (Residues 441 and 442)[a]	LKIAGA↓FGFK
(Residues 487 and 488)	GDEAQA↓ASGT
pVP2 (Residues 494 and 495)	SGTARA↓ASGK
(Residues 501 and 502)	SGKARA↓ASGR
pVP2-VP4 (Residues 512 and 513)	RQLTLA↓ADKG
VP4-VP3 (Residues 755 and 756)	YHLAMA↓ASEF

[a]Residues 441 and 442 are cleaved by the endopeptidase activity of pVP2 (Irigoyen *et al*., 2009).

Fig. 3A, the synthetic peptides were processed at a pH range of 7–9 and the reaction had the highest observed rate at pH 8.8. Therefore, all further kinetic determinations were carried out at pH 8.8 where VP4 displays an optimal activity.

The effect of temperature on the thermostability of VP4 tubules was shown in Fig. 3B. An increment in activity could be observed when the incubating temperature was between 40 and 60°C. While the temperature reached 70°C, the activity started to decline. The denatured VP4 aggregated after incubation at 80 and 90°C, and therefore the activity significantly dropped. Although the increase of activity with temperature is inevitable due to basics laws of physics and is limited by thermal denaturation, these results also revealed that VP4 tubules were relatively stable even when the temperature was raised to 70°C, which is a favorable temperature for industrial demands (Iyer and Ananthanarayan, 2008).

Divalent metal ions are often used as cofactors for enzyme activity, which prompted our further analysis to understand the effect of divalent metal ions on VP4 activity. Addition of Mg^{2+} to the reaction solutions did not increase the catalytic rate of VP4 significantly. In contrast, upon the additions of Ca^{2+}, Mn^{2+} and Ni^{2+} ions, inhibitions of catalysis were observed (Fig. 3C), especially for Ni^{2+}, which blocked the VP4 activity completely when its concentration reached 1 mM (Figs 3C–D). Inhibition of VP4 activity through the addition of Ni^{2+} ions has not been reported so far.

Steady-state kinetic constants of VP4 and mutants

From three separate experiments, the steady state kinetic constants for the VP4-catalyzed cleavage of the peptide K(Dabcyl)GKARAASE(Edans)G at pH 8.8 and 37°C are $k_{cat} = 0.04 \pm 0.01\ min^{-1}$, $K_m = 43 \pm 2\ \mu M$, and $k_{cat}/K_m = 926\ M^{-1}\ min^{-1}$ (or $\sim 15.4\ M^{-1}\ s^{-1}$). The activity of the expressed IBDV VP4 is lower than that of BSNV VP4 which has the k_{cat}/K_m value (at 25°C) of $41–166\ M^{-1}\ s^{-1}$ (Ekici *et al.*, 2009), and is much lower than that of the *E. coli* Lon protease with the k_{cat}/K_m value of $2–88 \times 10^3\ M^{-1}\ s^{-1}$ when a set of synthetic peptides were used as substrates (Patterson-Ward *et al.*, 2009). The low activity caused by a background signal was ruled out by a control experiment which showed no fluorescence change in the absence of VP4. To test whether the activity is produced by the *E. coli* protease contaminants, we constructed three active site mutants, VP4-S652A, VP4-K692A and VP4-S652A.K692A.

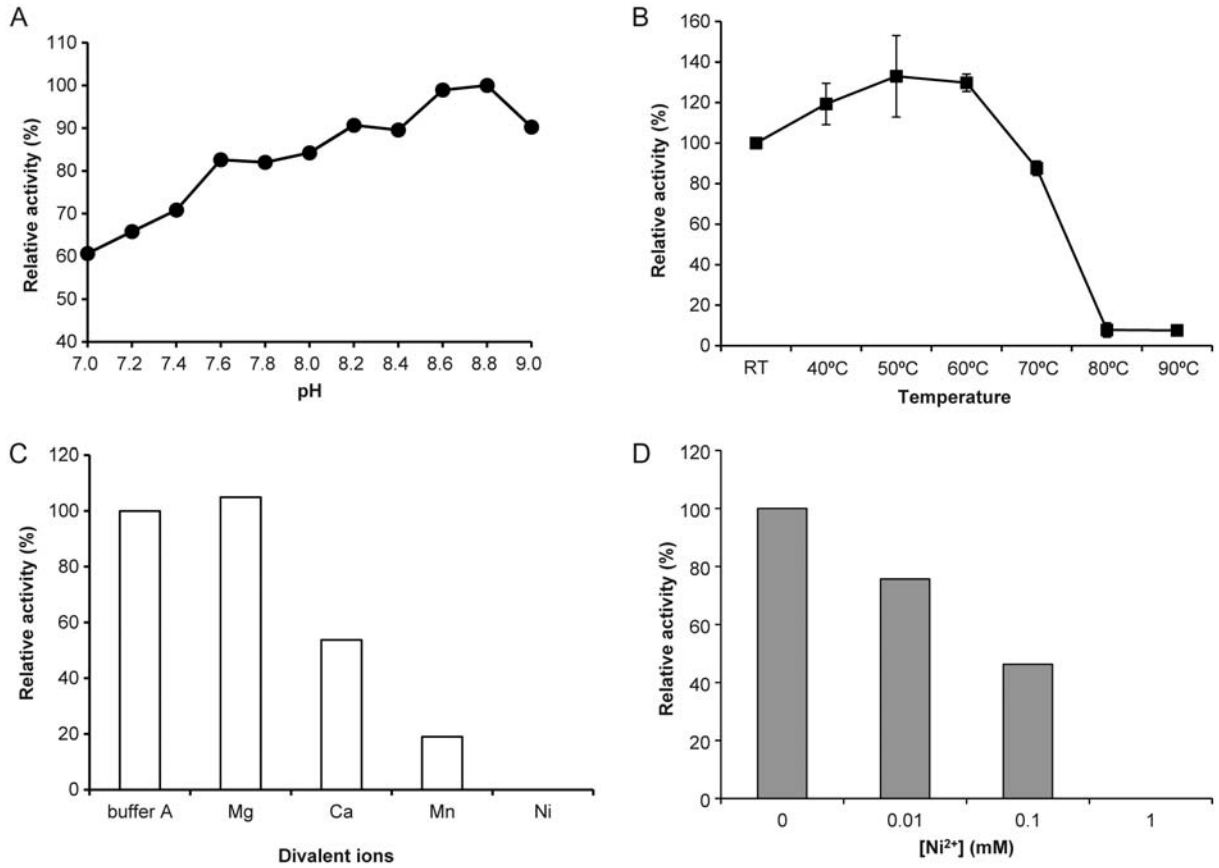

Fig. 3. Effect of pH, thermostability and cations on VP4 activity (**A**) Relative activity at different pHs. VP4 protease activity expressed as relative fluorescence unit (RFU) versus time was conducted in buffer A as described in Materials and methods. The relative activity was defined as the ratio of the endopeptidase activity at a specific pH to that at pH 8.8. (**B**) Relative activity after heat treatment at various temperatures. The reactive velocity in buffer A (pH 8.8) at room temperature without heat treatment (RT, room temperature) was defined as 100%. (**C**) The effect of divalent metal ions on VP4 activity. The reactive velocity in buffer A (pH 8.8) was defined as 100%. The velocities of reactions upon the addition of 10 mM of Mg^{2+}, Ca^{2+}, Mn^{2+} and Ni^{2+} were compared. (**D**) Dose-dependent inhibition of Ni^{2+} ion on VP4 protease activity.

The k_{cat} values for the three VP4 mutants to catalyze the cleavage of peptide K(Dabcyl)GKARAASE(Edans)G at pH 8.8 and 37°C are two to four times lower than that of the wild-type VP4 tubules (Table II). Our result is different from a previous report where replacement of the catalytic dyad residues (Ser-652 and Lys-692) with alanine completely inactivated polyprotein processing *in vivo* (Lejal *et al.*, 2000); however, the *in vitro* endopeptidase activity of the active site mutants has not been reported. In this study, although a total activity lost was expected for the three active-site mutants, the significant reduction in k_{cat} still suggested that S652 and K692 are crucial for the endopeptidase activity or IBDV polyprotein processing. In summary, the wild-type VP4 tubules have an enzymatic activity because its activity is higher than that obtained for any one of the mutants even if some activities are contributed by the contaminants.

Table II. Summary of the kinetic parameters of VP4 proteases

Protein	V_{max} (μM/min)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}.\ min^{-1}$)
VP4	0.14 ± 0.03	43 ± 2	0.04 ± 0.01	926 ± 149
S652A	0.04 ± 0.01	40 ± 2	0.01 ± 0.00	306 ± 76
K692A	0.06 ± 0.00	33 ± 10	0.02 ± 0.00	549 ± 158
S652A.K692A	0.03 ± 0.01	36 ± 10	0.01 ± 0.00	244 ± 1

The kinetic parameters were calculated by fitting data to the Michaelis-Menten equation.

Discussion

IBDV VP4 protein catalyzes the hydrolysis of polyprotein (pVP2-VP4-VP3) to form subsequent VP2, VP4 and VP3. This action catalyzed by VP4 is a crucial step for viral assembly and replication. The 3D structure of the protein may provide useful knowledge for antiviral strategy. Although IBDV VP4, like other VP4s of birnaviruses, uses Ser/Lys catalytic dyad mechanism, its protein fold near the active site may be predictable; however, due to the low-level identity of the primary amino acid sequences among these VP4s, the detail information on IBDV VP4 protein structure awaits to be obtained. This is the first report on the expression and purification of IBDV VP4 tubular endopeptidase from *E. coli* BL21(DE3)pLys culture. High expression level and successful purification of functional VP4 tubular endopeptidase demonstrated in this study can be applied to a large-scale preparation of VP4 tubules for structure determination.

Our FRET-based study on the activity of VP4 indicated that the expressed VP4 protein in a tubular form has an endopeptidase activity of k_{cat}/K_m ~15 $M^{-1}\ s^{-1}$ toward the synthetic peptide K(Dabcyl)GKARAASE(Edans)G. The tubular form of VP4 may explain its low level of k_{cat} (about sixty-

fold lower) when compared with that of the *E. coli* tail-specific protease, a member of the sub-family of serine proteases that utilize a Ser-Lys dyad as the principal catalytic machinery (Beebe *et al.*, 2000). This result confirmed that the formation of VP4 tubules may inactivate excess protease to prevent lethal damage to the virus or to the cells in IBDV-infected cells (Tacken *et al.*, 2003). Interestingly, this strategy was not reported for other members of birnaviruses.

In conclusion, we have successfully expressed the functional VP4 protein in the *Escherichia coli* strain BL21(DE3)pLys. The purification steps developed here contained an IMAC and a Sepharose gel filtration chromatography. This system provides efficient VP4 preparation methods, and can be also used for the preparation of VP4 mutants useful for structure–function studies and for the development of anti-IBDV drugs.

Supplementary data

Supplementary data are available at *PEDS* online.

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