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# Phospholipase $A_2$-like activity of human bocavirus VP1 unique region ☆

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**Abstract**

Human bocavirus (HBoV) is a new parvovirus first discovered in 2005, which is associated with acute respiratory infection. Analysis of sequence homology has revealed that a putative phospholipase $A_2$ ($PLA_2$) motif exists in the VP1 unique region of HBoV. However, little is known about whether the VP1 unique region of HBoV has $PLA_2$ enzymatic activity and how these critical residues contribute to its $PLA_2$ activity. To address these issues, the VP1 unique region protein and four of its mutants, were expressed in *Eschericha coli*. The purified VP1 unique protein (VP1U) showed a typical $Ca^{2+}$-dependent secreted $PLA_2$-like ($sPLA_2$) activity, which was inhibited by $sPLA_2$-specific inhibitors in a time-dependent manner. Mutation of one of the amino acids (21Pro, 41His, 42Asp or 63Asp) in VP1U almost eliminated the $sPLA_2$ activity of HBoV VP1U. These data indicate that VP1U of HBoV has $sPLA_2$-like enzymatic activity, and these residues are crucial for its $sPLA_2$-like activity. Potentially, VP1U may be a target for the development of anti-viral drugs for HBoV.


*Keywords:* Human bocavirus; VP1 unique region; Phospholipase $A_2$; Point mutation

Human bocavirus (HBoV) was identified in clinical specimens from infants and children suffering from respiratory infection in 2005 [1]. Recent studies have shown that HBoV infection accounts for 1.5–11.3% of respiratory infectious diseases in North America [2,3], Europe [4,5], Asia [6,7], Australia [8] and Africa [9], which suggests that infection with the virus is a global problem. HBoV is prevalent among children with acute wheezing, asthma, bronchiolitis, pneumonia and possibly other systemic infectious diseases [10–14].

Phylogenetic analysis of the complete genome of HBoV reveals that the virus is closely related to canine minute virus (CMV) and bovine parvovirus (BPV), which are members of the *Bocavirus* genus of the *Parvoviriade* family, subfamily *Parvovirinae*. HBoV has two major ORFs, which encode a nonstructural protein (NS1) and at least two capsid proteins (VP1 and VP2). Like other members in the *Parvovirinae* [1,15], the capsid proteins, VP1 (671 aa) and VP2 (542 aa), have the same sequence of 542 aa at the C termini, while VP1 has an additional 129-aa peptide at the N terminus.

Recently, a conserved phospholipase $A_2$ ($PLA_2$) motif, which resembles the catalytic motif of secreted $PLA_2$ ($sPLA_2$), has been identified in the VP1 unique region (VP1U) of most parvoviruses [16–21]. Phospholipases are enzymes that hydrolyze phospholipids into free fatty acids and lysophospholipids. The $sPLA_2$ hydrolyzes specifically the 2-acyl ester (*sn*-2) bond of phospholipid substrates into lysophopholipids and free fatty acids. The $sPLA_2$-like activity in parvovirus is thought to be critical for the efficient transfer of the viral genome from late endosomes/lysomes to the nucleus for initiating viral replication [18,20]. A single amino acid substitution in the active site of the $sPLA_2$ motif of VP1U inactivates enzymatic activity and eliminates virus infectivity [20,21]. Analysis of the

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VP1U sequence of HBoV reveals a conserved HDXXY motif in the catalytic center of $sPLA_2$. Furthermore, the YXGXG motif, the conserved $Ca^{2+}$ binding loop of sPLA2, exists in the VP1U sequence of HBoV and other parvoviruses. According to homology studies, a putative $sPLA_2$ motif is present in the VP1U of HBoV, which spans positions 21–63. The amino acid residue at position 21 (Pro) is thought to be important for binding calcium ions while residues 41(His), 42(Asp) and 63(Asp) are proposed to form the catalytic network for the enzymatic activity, similar to other parvoviruses [20]. However, whether VP1U of HBoV has phospholipase activity is still unclear. If it does, how these critical amino acid residues contribute to its activity remains to be determined.

Our previous studies have shown that HBoV infection accounts for 8% of respiratory infections in Chinese children (7). To further characterize the VP1U of HBoV isolated from China, the genes for the VP1U segment and its four mutants (P21R, H41A, D42N or D63A) were generated and expressed in *Escherichia coli*. Their enzymatic $sPLA_2$-like activities and their calcium-dependence were analyzed using the $PLA_2$ enzymatic assays and $sPLA_2$-specific inhibitors.

## Materials and methods

*Cloning and site mutagenesis of the VP1U gene*. The gene for VP1U of HBoV was designed with optimized codon usages and synthesized (Generay Biotech, Shanghai, China), according to the codon usage bias in *E. coli* (http://www.kazusa.or.jp/codon/). The gene with optimized DNA sequence was amplified by PCR using sense primer 5′-CAT ATG CCC CCT ATC AAA CGT C-3′ (added NdeI restriction site) and anti-sense primer 5′-CTC GAG TTT CGA GGT CCC TGG TTT CG-3′ (added XhoI restriction site). The produced DNA fragment was cloned into prokaryotic expression vector pET30a(+) (Novagen) with a 6-His tail at the C terminus. To generate VP1U mutants, the cloned DNA was subjected to site-mutagenesis by changing the amino acid residue at position 21 (Pro), 41(His), 42 (Asp) or 63 (Asp) of the VP1U into arginine (P21R), alanine (H41A), asparagine (D42N) or alanine (D63A), respectively, in the sPLA2 motif of VP1U (Fig. 1), using the QuickChange Site-directed Mutagenesis Kit in accordance with the manufacturer's instructions (Stratagene). Their authenticity was analyzed by DNA sequencing.

*Expression and purification of VP1U protein*. To express VP1U and its mutants, their plasmids were transformed into *E. coli* BL21 (DE3) cells. The cells were grown in 1 l LB medium containing kanamycin (50 μg/ml) up to $OD_{600}$ 0.8, and induced with 1 mM isopropyl-thio-D-galactopyranoside (IPTG) at 37 °C for 4 h. The cells were pelleted and re-suspended in 40 ml washing buffer (10 mM Tris/HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100), followed by sonication. Their inclusion bodies were collected by centrifugation at 12,000*g* for 20 min and washed four times with washing buffer. The resulting pellets were solubilized in 20 ml lysis buffer (6 M guanidine/HCl, 10 mM Tris/HCl, 0.2 M NaCl, pH 8.0). The solubilized inclusion bodies were centrifuged at 12,000*g*, 4 °C for 30 min. The supernatants were filtered through a 0.22-μm syringe filter and loaded on a $Ni^{2+}$ chelating column for the purification of these proteins, according to the manufacturer's instruction (GE Healthcare). The proteins of interest were eluted from the columns and concentrated using the centrifuging filter units (3-Kd cut-off). Protein concentrations were determined using the BCA protein assay kit (Pierce) and these proteins were analyzed by 12% SDS–PAGE. Protein N-terminal sequencing was performed in an ABI Procise 492cLC sequencer (Genecore, Shanghai, China).

*sPLA2 catalytic activity assay*. The sPLA2 activity of VP1U protein and its mutants (P21R, H41A, D42N, D63A) was assayed using the sPLA2 Activity Kit (Cayman Chemical, Ann Arbor, MI, USA) and the 1,2-dithio analog of diheptanoyl phosphatidylcholine (Diheptanoyl Thio-PC) as the substrate for $PLA_2$, in accordance with the manufacturer's instructions. Upon hydrolysis of the thioester bond at the *sn*-2 position by $PLA_2$, free thiols were detected using 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) with dynamic colorimetric measurements at 405 nm every minute for 10 min. The VP1U, P21R, H41A, D42N or D63A (100 ng, each) as well as a positive control of bee venom $PLA_2$ (5 ng) was tested for three times and analyzed at five different time-points. Results are expressed as nmol/min/ml.

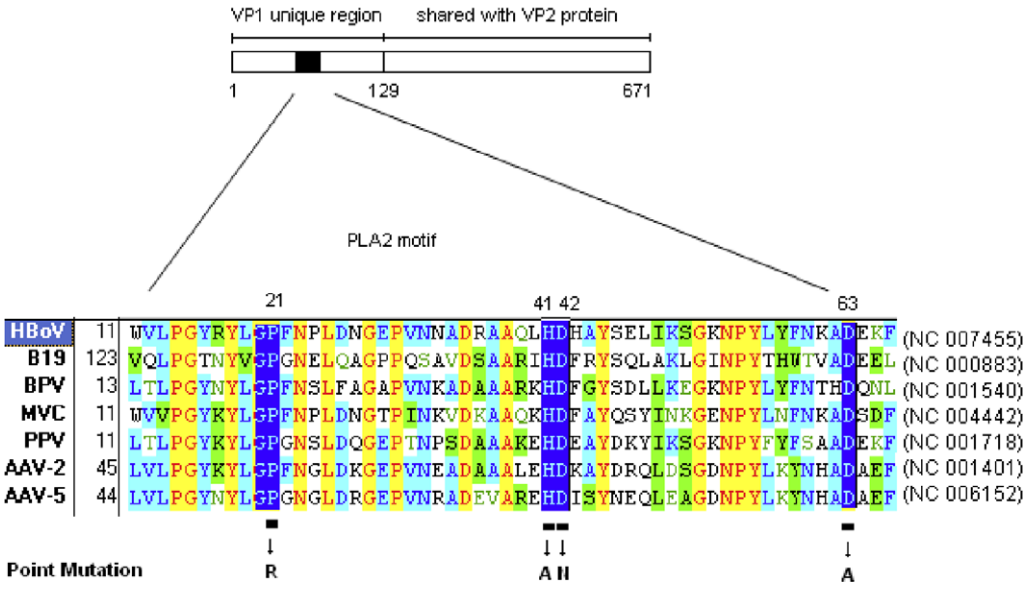


Fig. 1. Schematic illustration of the HBoV VP1/VP2 region. The putative $PLA_2$ motif located in the VP1 unique region is indicated by the amino acid sequence. The potential catalytic site and calcium-binding site (shaded gray) are indicated. The four mutants (P21R, H41A, D42N, and D63A) are indicated (arrows).

*Calcium association studies*. To examine the influence of calcium on $sPLA_2$ activity, the $PLA_2$ activity of VP1U protein (100 ng) and bee venom PLA2 (5 ng) was examined in the presence or absence of 10 mM $CaCl_2$.

*$PLA_2$ inhibition assay*. Two $PLA_2$ inhibitors, manoalide (MLD; Sigma) and 4-bromophenacylbromide (4-BPB; Sigma), were used to examine their specific inhibition of the $sPLA_2$ enzymatic activity of VP1U. VP1U (500 ng) or bee venom PLA2 (5 ng) was pre-incubated with 50 mM MLD or 4-BPB (dissolved in DMSO), respectively, at 37 °C and $sPLA_2$ activity was measured after 0, 1, 2, 3, 4 or 5 h. The VP1U (500 ng) or bee venom $PLA_2$ (5 ng) incubated without inhibitor for 5 h were used as negative controls.

## Results

### *Cloning, mutagenesis, expression and purification of VP1U protein*

To characterize the VP1 unique region of HBoV isolated in China, the DNA segment for the VP1U was synthesized with optimized code sequences and cloned into the expression vector pET30a(+). After DNA sequencing, the plasmid was transformed into *E. coli*. Following induction, a fusion VP1U protein (129 aa) containing a 6× His tail at the C terminus was achieved. To further investigate the role of critical amino acids in the VP1U $sPLA_2$ motif in enzymatic activity, four mutants (P21R, H41A, D42N, D63A) were generated by site-directed mutagenesis (Fig. 1), and cloned into pET30a(+). After DNA sequencing, these plasmids were transformed into *E. coli* BL21 (DE3) strain, and their protein expression was induced with 1 mM IPTG, and characterized by SDS–PAGE. As expected, these VP1U and mutant proteins were mainly located in cell inclusion bodies, with an approximate molecular mass of 14 kDa, according to a protein standard marker (data not shown). To obtain purified proteins, the inclusion bodies were washed and sonicated, followed by purification using the NTA–Ni affinity column. The purified proteins were characterized by SDS–PAGE and reached purity >95% (Fig. 2). The N-terminal 10 aa were analyzed by protein sequencing. The success of cloning, expression and purification of those VP1U and mutants provides a basis for the characterization of their enzymatic activity and critical residues for their $sPLA_2$ function.

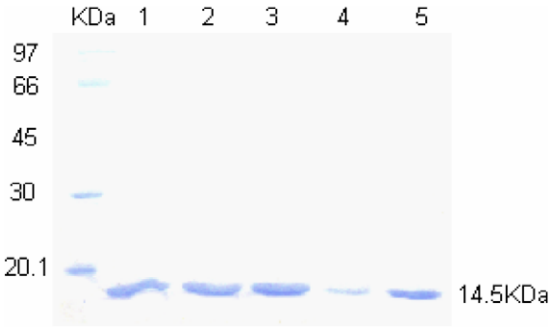


Fig. 2. SDS–PAGE analysis of purified proteins. Lane 1, VP1U protein; lane 2, P21R; lane 3, H41A; lane 4, D42N; and lane 5, D63A.

### *VP1U protein has $sPLA_2$ activity*

To determine the $sPLA_2$ activity of the VP1U generated in *E. coli*, the purified VP1U (100 ng) and bee venom $PLA_2$ (5 ng) were characterized for their $PLA_2$ activity in the presence of 10 mM calcium. Both the purified VP1U and bee venom $PLA_2$ showed $PLA_2$-like activity, however, their $PLA_2$ activities were different. The $sPLA_2$ activity of VP1U was $49.2 \pm 3.6$ (means ± SD) nM/min/ml, which was similar to that of human adeno-associated virus [19]. In contrast, the activity of bee venom $PLA_2$ reached $201 \pm 7.6$ (means ± SD) nM/min/ml (Fig. 3). Therefore, the purified VP1U of HBoV had a lower level of $sPLA_2$-like activity.

### *VP1U $sPLA_2$-like activity is calcium-dependent*

The $sPLA_2$-like activity of VP1 proteins from known parvoviruses is calcium-dependent. We examined the influence of calcium on the $sPLA_2$ activity of HBoV VP1U in the presence or absence of $Ca^{2+}$. The $sPLA_2$ activity of VP1U in the presence of 10 mM $Ca^{2+}$ reached $49.2 \pm 3.6$ (means ± SD) nM/min/ml, while the $sPLA_2$ activity in the absence of calcium was reduced by 84% to $8.15 \pm 0.6$ (means ± SD) nM/min/ml (Fig. 3). Similarly, the bee venom $PLA_2$ activity in the absence of calcium significantly decreased by 40%, as compared with that in the presence of calcium. The significantly reduced $sPLA_2$ activity in the absence of calcium suggests that the $sPLA_2$-like activity of the VP1U protein, like the bee venom $PLA_2$, is calcium-dependent.

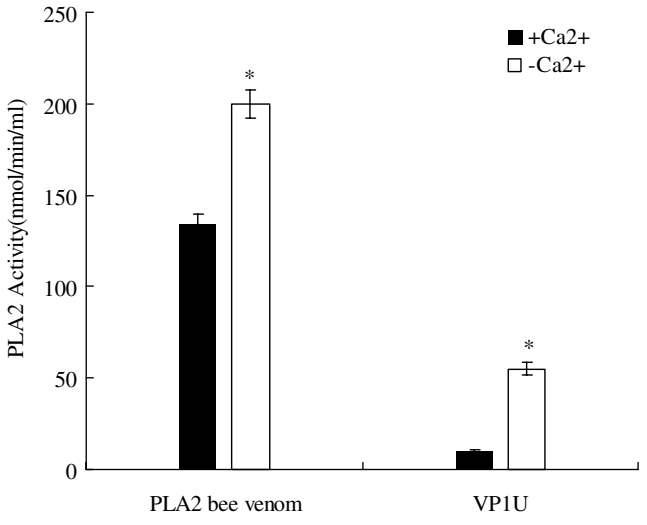


Fig. 3. $PLA_2$ activity of VP1U protein. The PLA2 activity of VP1U (100 ng) and bee enom $PLA_2$ (5 ng) was characterized in the presence or absence of 10 mM calcium. Data are presented as the means ± SD of three separate experiments. $^*P < 0.01$, as compared to the value in the absence of calcium.

### *sPLA$_2$ activity of VP1U is inhibited by MLD or 4-BPB*

MLD or 4-BPB covalently attaches to lysine or histidine residues in the catalytic center or the phospholipid binding site of the PLA$_2$ enzyme, and inhibits its enzymatic activity [22,23]. To further demonstrate the sPLA$_2$-like activity of VP1U, two specific sPLA$_2$ inhibitors, MLD, and 4-BPB, were used to inhibit the sPLA$_2$-like activity of VP1U. VP1U and control bee venom PLA$_2$ were pre-incubated with, or without, MLD or 4-BPB for different times, and their PLA$_2$ activity was characterized. MLD or 4-BPB inhibited the PLA$_2$ activity of VP1U and bee venom PLA$_2$ in a time-dependent fashion. The PLA$_2$ activity of VP1U or bee venom PLA$_2$ cultured alone for 5 h was similar to that of freshly prepared enzyme, which suggests that incubation of VP1U or bee venom PLA$_2$ for 5 h did not affect PLA$_2$ activity (Fig. 4). One or two hours after incubation with MLD or 4-BPB, PLA$_2$ activity of VP1U decreased by 25% or 50%, respectively. However, bee venom PLA$_2$ appeared to be more sensitive, as the PLA$_2$ activity of bee venom PLA$_2$ cultured with 4-BPB or MLD for 1 h was reduced by 60% or 50%, respectively. Furthermore, MLD showed a slightly higher inhibitory effect on both the VP1U and bee venom PLA$_2$ activity. Hence, the sPLA$_2$-like activity of VP1U is inhibited by sPLA$_2$-specific inhibitors MLD and 4-BPB.

### *Mutation of critical residues in VP1U abrogates its sPLA$_2$ activity*

Amino acid residue 21(Pro) is thought to be important for binding calcium ions, and residues 41(His), 42(Asp), and 63(Asp) have been proposed to form the catalytic network for enzymatic activity (20). To demonstrate their role, four mutants (P21R, H41A, D42N or D63A) were generated and their PLA$_2$ activity is shown in Table 1. All four

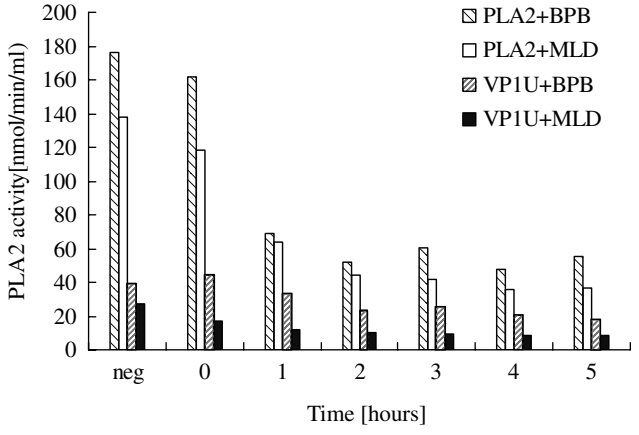


Fig. 4. Inhibition of PLA$_2$ activity of VP1U. The VP1U (100 ng) and bee venom PLA$_2$ (5 ng) were incubated with 50 μM MLD or 4-BPB for the indicated time and their PLA$_2$ activity was determined. VP1U or bee venom PLA$_2$ was incubated for 5 h in the absence of any PLA$_2$ inhibitor and used as the negative control (neg). Data are representative of three repeated experiments.

Table 1
The sPLA$_2$-like activities of VP1U and mutants

| Protein | Mutagenesis site | Relative activity (%) |
|---|---|---|
| VP1U | — | 100 |
| P21R | Pro→Arg at aa 21 | 1.05 |
| H41A | His→Ala at aa 41 | 0.52 |
| D42N | Asp→Asn at aa 42 | 1.48 |
| D63A | Asp→Ala at aa 63 | 0.64 |

mutants showed much lower levels of sPLA$_2$ activity. Replacement of 21(Pro) with Ala reduced sPLA$_2$-like activity by >90%, while mutation of H41, D42 or D63 dramatically mitigated sPLA$_2$-like activity, as compared with that of parent VP1U protein. The abrogation of sPLA$_2$-like activity in these mutants demonstrates that these residues are critical for the sPLA$_2$-like activity of HBoV VP1U.

## Discussion

Recently, a conserved PLA$_2$ motif has been identified by sequence alignment in the VP1 unique region of most parvoviruses. HBoV is a newly discovered parvovirus and its VP1 unique region contains the putative PLA$_2$ motif. In addition, amino acid position P21 in the YXGPG sequence in the $Ca^{2+}$ binding loop is strictly conserved, which resembles other parvoviruses [20], but is different from sPLA$_2$.

To determine whether this putative PLA$_2$ domain has PLA$_2$-like activity, the gene for VP1U protein was designed and synthesized with optimized codon usages, according to the codon bias of *E. coli*. The VP1U protein was overexpressed in the inclusion bodies of strain BL21 (DE3) and further purified using NTA–Ni affinity chromatography, which reached a purity >95%. The purified VP1U protein had sPLA$_2$-like activity, similar to that in other parvoviruses. However, the sPLA$_2$ activity of HBoV VP1U was ~100-fold lower than that of bee venom PLA$_2$. In a previous study, the specific activity of three viral PLA$_2$ differed by three orders of magnitude. The PLA$_2$ activity of porcine parvovirus is similar to that of the most active sPLA$_2$s, while PLA$_2$ activity of human AAV2 and B19 parvovirus are ~$10^3$ times lower than that of sPLA$_2$ [17]. Our data showed that the sPLA$_2$ activity of HBoV VP1U is similar in magnitude to that of human AAV2.

The sPLA$_2$-like activity of HBoV VP1U was inhibited by the PLA$_2$-specific inhibitors MLD or 4-BPB, in a time-dependent manner, similar to B19 VP1U [19]. Following incubation with MLD or 4-BPB for 1 h, VP1U sPLA$_2$-like activity decreased by 25%, and extending the incubation to 3 h, MLD or 4-BPB inhibited the sPLA$_2$-like activity by nearly 50%. Notably, treatment of the VP1U or bee venom PLA$_2$ with 4-BPB for 3 h slightly increased their PLA$_2$ activity. This may be due to delayed access to the catalytic site of the enzyme by the substrate, or by the additional C-terminal protein domain in VP1U [19,23]. Alternatively, additional histidine residues (His 43, His 105)

and the C-terminal fusion 6× His epitope in VP1U may intercept the inhibitor molecule, and delay its inhibitory effect.

Analysis of protein structure revealed that the $sPLA_2$ motif of parvovirus has the $Ca^{2+}$ binding site, YXGPG. Our data showed that the $sPLA_2$-like activity of HBoV VP1U was $Ca^{2+}$-dependent, similar to that of almost all known parvoviruses [17,20]. Indeed, the $sPLA_2$-like activity of HBoV VP1U in the absence of $Ca^{2+}$ was reduced by 82%, compared with that in the presence of $Ca^{2+}$. Importantly, mutant P21R, which changes the conserved proline to arginine in the $Ca^{2+}$-binding loop of all parvovirus $PLA_2$s [19–21], almost completely abrogated the $sPLA_2$-like activity of HBoV VP1U. Interestingly, mutagenesis of one of the other three residues in VP1U, which are proposed to constitute the active network of $PLA_2$, reduced the $sPLA_2$-like activity of HBoV VP1U by 99%. Conceivably, these mutants not only changed the acceptance of VP1U for $Ca^{2+}$-binding and constitution of enzymatic sites, but also modulated the conformation of VP1U. Therefore, the native conformation of VP1U is necessary not only for $Ca^{2+}$ binding, but also for the externalization of inner VP1U in parvovirus to expose the active center [24–26].

Parvovirus $PLA_2$ activity has been shown to be responsible for mediating efficient transfer of the viral genome from late endosomes/lysosomes to the nucleus for initiating viral replication. Furthermore, recent studies have shown that parvovirus $PLA_2$ activity may activate $I_{CRAC}$, which participates in the pathophysiological process of B19 infection and non-infectious B19 [16]. In addition, the $PLA_2$ activity of B19 VP1U can activate synoviocytes and up-regulate cyclooxygenase expression, which contribute to synovial inflammation [27]. Conceivably, HBoV VP1U may have similar functions, contributing to the pathogenesis of HBoV infection.

In summary, the gene for HBoV VP1U was successfully cloned and expressed. Purified VP1U showed $sPLA_2$-like activity, which was $Ca^{2+}$-dependent and sensitive to $PLA_2$-specific inhibitors. Amino acid 21Pro, 41His, 42Asp or 63Asp was crucial for the $PLA_2$-like activity of HBoV VP1U. Given that the $sPLA_2$-like activity of VP1U of parvoviruses has been shown to participate in the pathogenesis of parvovirus infection, our data may provide new insights into the pathogenesis of HBoV infection in humans. Potentially, VP1U may be a target for the development of antiviral drugs for HBoV infection.

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