Proteases in *Vibrio cholerae* and Their Role in Pathogenesis

Amit Pal and Sun Nyunt Wai

Abstract Hemagglutinin protease (HAP) produced by *Vibrio cholerae* is the most well characterized protease in pathogenic Vibrio. The matured 45-kDa and 35-kDa processed forms of HAP were purified from a *ctx* gene negative *Vibrio cholerae* O1 strain. The 35-kDa HAP showed hemorrhagic fluid response in a dose dependent manner in the rabbit ileal loop assay (RIL). Histopthological examination of purified protease treated rabbit ileum showed the presence of erythrocytes and neutrophils in the upper part of the villous lamina propria, gross damage of the villous epithelium with inflammation, hemorrhage and necrosis. The 35-kDa form of HAP, when added to the luminal surface of the rat ileum loaded in an Ussing chamber, showed a decrease in the intestinal short-circuit current and a cell rounding effect on HeLa cells. Almost all results of earlier studies suggest an indirect pathogenic role of HAP but this study showed the possibility of a direct role of HAP in pathogenesis. The other well characterized protease secreted by *Vibrio cholerae* is PrtV a metalloprotease which has a role in the protection from predator grazing in natural aquatic environments and also has a role in human pathogenicity. PrtV also modulated hemolysin which plays a role in inflammatory response in human epithelial cells. A novel 59-kDa serine protease was identified from a Δ*hapA*Δ*prtV V. cholerae* O1 strain. The serine protease has been shown to cause hemorrhagic fluid response in RIL assay. Proteases secreted in *Vibrio cholerae* play a role in its pathogenesis.

Keywords *Vibrio cholerae* pathogenesis • Hemagglutinin protease (HAP) • *Vibrio cholerae* protease (PrtV) • 59-kDa Serine protease

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1 Introduction

The major features of pathogenesis of cholera are well established. Infection due to *Vibrio cholerae* begins with the ingestion of contaminated water or food. After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of the toxin-coregulated pili [1] and possibly other colonization factors such as the different hemagglutinins, accessory colonization factor and core-encoded pilus, all of which are thought to play a role. Cholera enterotoxin produced by the adherent *vibrios* is secreted across the bacterial outer membrane into the extracellular environment and disrupts ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes leads to severe diarrhea which is characteristic of cholera [2].

Proteases produced by pathogenic microorganisms play an important role in virulence [3]. Proteases are enzymes that catalyze the hydrolysis of peptide bonds in proteins or peptides. They are either exopeptidases, whose actions are restricted to the N- or C-termini of proteins, or endopeptidases which cleave internal peptide bonds. Microbial peptides are predominantly secreted enzymes and can be classified based on the essential catalytic residue at their active site. They include serine proteases, cysteine proteases, aspartate proteases and metalloproteases. Tissue barriers to pathogen invasion, such as extracellular matrices, epidermal keratinocyte layers and blood vessel walls, may be targeted by bacterial proteases. Proteolysis of host tissue components such as extracellular matrix proteins, including collagen, laminin, fibronectin and elastin, can induce necrotic tissue damage which may aid the bacteria in host cell entry [4, 5]. *V. cholerae* O1, the causative agent of epidemic cholera, secretes a 32-kDa zinc-containing hemagglutinin protease. Most results suggest that HAP plays an indirect role in pathogenesis. Our study is the first study to show direct role of HAP in pathogenesis of *V. cholerae* [6]. A metalloprotease, other than HAP, *Vibrio cholerae* protease (PrtV) plays a role in protection from predator grazing in natural aquatic environments. Using a Δ*hapA*Δ*prtV V. cholerae* strain a novel 59 kDa serine protease was identified and shown to play a role in hemorrhagic response in rabbit ileal loop [7].

2 Proteases in *Vibrio cholerae*

2.1 *Hemagglutinin Protease*

V. cholerae O1 El Tor biotype strains, as well as the non-O1, non-O139 serogroup strains produce several hemagglutinins [8–11] which may potentially be involved in adherence of the vibrios to the human gut. In particular, the soluble hemagglutinin, which also has proteolytic activity [12] is called hemagglutinin/protease (HAP). HAP (vibriolysin) belongs to a family of metalloprotese (M4: M04/003) which is largely distributed among pathogenic bacteria. Comparison of multiple

sequences shows considerable sequence similarity among primary structures of all known neutral proteases of M4 family. The active site and metal binding residues are highly conserved. HEXXH is the putative signature of Zn-metalloprotease. This motif contains all of the residues that have been positively identified as part of the active site [13, 14].

When HAP is purified in the presence of EDTA, a larger 45-kDa form is isolated while in absence of EDTA, a 32-kDa form is purified [6]. The protease undergoes several steps of processing, including cleavage of the signal peptide and further processing of the N terminus and the C terminus to generate the mature protein [14]. The 45-, 37-, 32-, and 9-kDa bands are the major bands bound to anti-HAP antibodies, which suggests that they are the proteolytic degradation products of the 45-kDa polypeptide [15]. Purified HAP in the absence of EDTA from *V. cholerae* non-O1, non-O139 strain PL-21 in TSB medium showed the presence of both 45- and 35-kDa forms [6]. In the presence of EDTA, the 45-kDa form of HAP was purified in larger amounts but in an in-active state. This is first study in which the two major forms of HAP were purified and studied in Ussing's chamber, rabbit ileal loop and tissue culture assays. The functional activity of HAP is related to its protease activity, and HAP being a metalloprotease, it shows no protease and HA activity in the presence of EDTA. The mature 45-kDa form of HAP purified in the presence of EDTA showed no effects in RIL, Ussing chamber, and tissue culture assays. Functional forms were generated when 45-kDa HAP was dialyzed in EDTA-free buffer. Once the protease is functional, it is processed, as is evident by the presence of 45, 42, and 35-kDa bands on SDS-polyacrylamide gels when HAP was purified in the absence of EDTA. *V. anguillarum* secretes a metalloprotease that also exists in two forms, Pa and Pb [16]. The Pb form was converted to the Pa form when the protease was incubated overnight at 4 °C. HAP exists in both the 45-kDa and 35-kDa forms but is not completely converted to the smaller processed form at 4 °C. Functional studies with only the 45-kDa form are not possible, as one cannot prevent its processing to the 35-kDa form in the absence of EDTA. However, the 45-kDa form can be studied through in vitro models such as Ussing chamber and tissue culture assays. This protein, at lower concentrations of 2 µg/ml and 4 µg/ml, exerts an enterotoxic response in the Ussing chamber in the form of an increase in I_{SC} (Fig. 1a) and a cell distending effect in the HeLa cell assay (Fig. 2b). At higher concentrations, it shows a decrease in the I_{SC} in the Ussing chamber (Fig. 1a), suggesting a cytotoxic response and a cell rounding effect on HeLa cells (Fig. 2a). The most probable explanation for this differential effect is that, at lower concentrations of functional 45-kDa HAP, the amount of 35-kDa form is less abundant, while the amount of the processed 35-kDa form is elevated at higher concentrations of 45-kDa HAP and may be higher than a critical level when it exerts a cytotoxic effect. The I_{SC} changes peaked at 4 µg/ml, while the effect observed at 6 µg/ml revealed that the enterotoxic response of the 45-kDa form was masked by the cytotoxic effect of the 35-kDa form, resulting in a decrease in I_{SC}. The enterotoxic response of the 45-kDa HAP started after a delay of 45–60 min and was equivalent to the effect of 1 µg/ml of CT in the rat small intestine [17]. Figure 1a shows that all protein concentrations of 45-kDa form upto 4 µg/ml the I_{SC} exhibited a gradual increase in
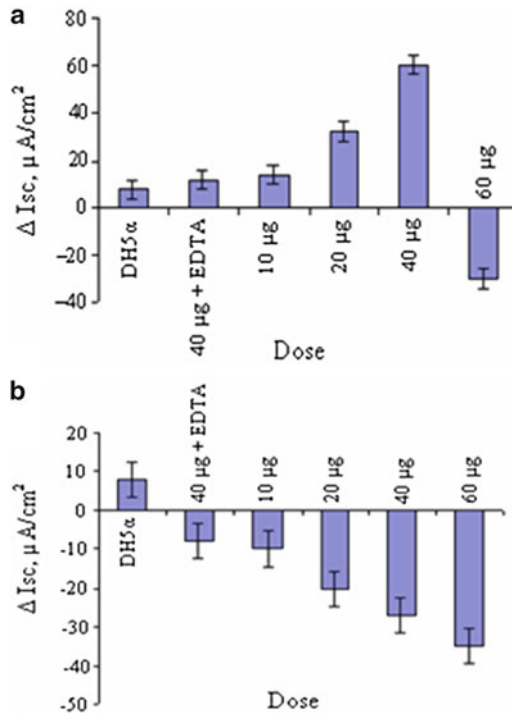
Fig. 1 Dose dependent response of HAP on intestinal short circuit current (Isc) of rat ileum loaded in Ussing's chamber. HAP was added in absence or presence of EDTA to the Ringer's solution in Ussing's chamber and the change in Isc was recorded for next 2 h from the time the sample was added. The data represents the mean and standard deviation for triplicate experiments. Proteins purified from DH5α were used as controls. (**a**). Response of Isc to increasing doses of 45-kDa HAP. (**b**). Response of Isc to increasing doses of 35-kDa HAP. There is an initial increase, but the Isc falls as the dose reaches beyond 40 µg/ml. Values are the mean standard deviations (error bars) for triplicate experiments. Proteins purified from E. coli DH5α were used as controls

rabbit ileal mucosa, with the peak being reached at different time intervals, depending on the protein concentration used, indicative of Cl⁻ secretion. The addition of glucose at the end of the experiment showed equivalent responses in treated and untreated tissue, indicating that glucose-Na⁺ active co-transport function was unaffected. Enterotoxicity is caused by an abnormal flux of negatively charged ions from the serosal side to the mucosal side [18]. Interestingly, at a higher concentration (6 µg/ml), there was a significant decrease in I_{SC} (Fig. 1a). The dose dependent effect of the purified 35-kDa HAP showed a sharp decrease in the intestinal short circuit current (Fig. 1b). The purified 35-kDa form of HAP also showed cell rounding effect (Fig. 2a). It was earlier reported that culture supernatant prepared from the reactogenic strains of *V. cholerae* cause a decrease in the transcellular epithelial resistance of cultured T84 cells [19]. This decrease correlated with the presence of HAP but not with the presence of other potential accessory toxins or proteases [19].
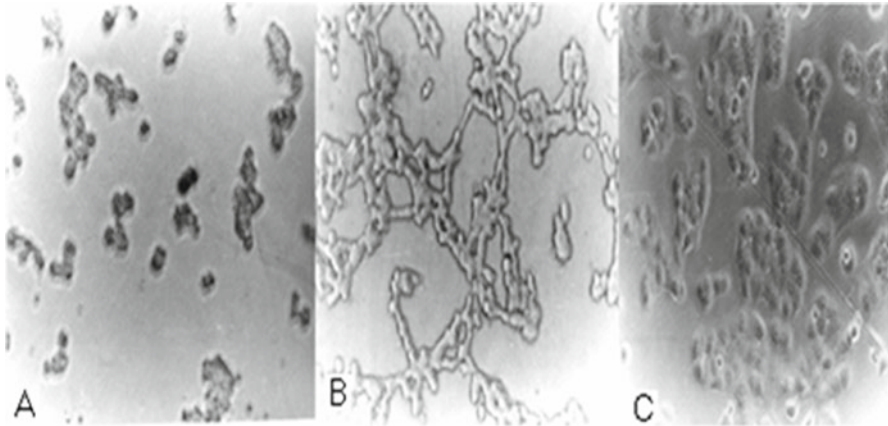
Fig. 2 Effects of HAP on HeLa cells. HeLa cells grown in tissue culture dishes were treated overnight with different concentrations of either the 35-kDa HAP or the 45-kDa HAP. Cells were observed under phase contrast microscope. (**a**). Cell rounding effect of 35-kDa HAP and higher concentration of 45-kDa HAP. (**b**). Cell distending effect of lower concentration of 45-kDa HAP (**c**). Normal HeLa cells grown in tissue culture plates

Purified HAP showed a dose-dependent hemorrhagic fluid response in the rabbit ileal loop assay. At 20 µg, a weak hemorrhagic response was elicited. At HAP concentrations increasing up to 60 µg there was a gradual increase in the fluid accumulation ratio, with hemorrhagic fluid present at all the different concentrations. The protease incubated in the presence of EDTA failed to show any response in RIL assay. The results of the RIL assay were substantiated by histopathological analysis of the ileal loop samples. In all the experiments, the rabbits were sacrificed 18 h postinoculation. The villous architecture and mucosa in the buffer control sections were normal (Fig. 3a). Purified HAP at a concentration of 20 µg resulted in little distortion of the villous pattern. The blood vessels were congested, and inflammatory cells were present in the lamina propia. High-powered photomicrograph showed the presence of erythrocytes in the upper part of the villous lamina propria. After treatment with 40 µg of purified HAP, there were marked alterations of the villous contour with red blood cell and inflammatory cell infiltrates in the villous epithelium and lamina propria and in damaged crypt epithelium (Fig. 3b). Higher magnification of portions of Fig. 3a, b revealed the clear presence of erythrocytes, eosinophils, and neutrophil polymorphs in the lamina propia (Fig. 3c, d). Washed *V. cholerae* 569B cultures, on the other hand, showed non-hemorrhagic fluid accumulation in the RIL assay. The active dialyzed 45-kDa form tested for entertoxigenicity in the RIL assay also showed hemorrhagic fluid response

HAP can proteolytically activate CT-A subunit [20] and the El Tor cytolysin/hemolysin [21] and can hydrolyze several physiologically important proteins such as mucin, fibronectin, and lactoferrin [22]. HAP perturbs the paracellular barrier of cultured intestinal epithelial cells [19, 23] by acting on tight-junction-associated proteins [23] and promotes the detachment of vibrios from monolayers and mucin [24, 25].
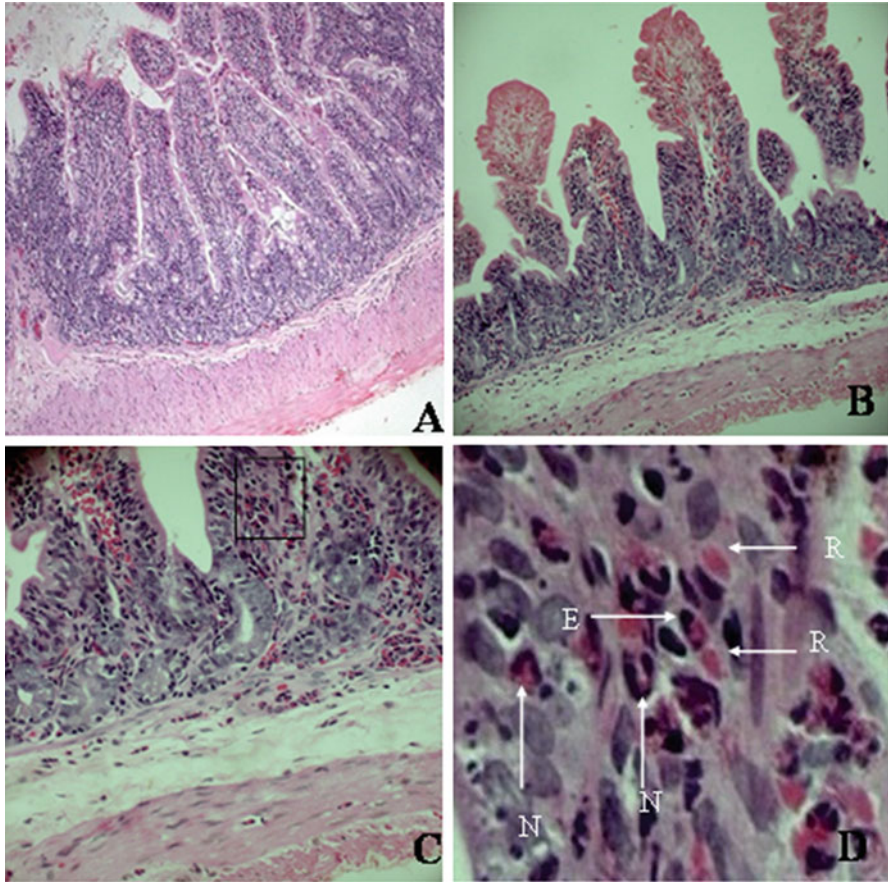
Fig. 3 Effect of purified HAP in the RIL assay. Purified HAP treated ileal tissues were processed for histopathological analysis and photomicrographs were taken. (**a**) Villus architecture observed in 25 mM Tris-HCl treated ileal tissues (10× magnification). Figure shows normal villous structure. (**b**) 40 μg of purified 35-kDa HAP treated rabbit ileal tissues show disruption of normal villus architecture with shortening of the villi (20× magnification). (**c**) 40× magnification of Fig. 4b shows infiltration of polymorphonuclear neutrophils, eosinophils and erythrocytes. (**d**) The *inset box* in Fig. 4c was further magnified (200×) to show the presence of RBC (*R*), neutrophils (*N*) and eosinophils (*E*). Magnification 200×

2.2   **Vibrio cholerae** *Protease (PrtV)*

The second most well characterized protease in *Vibrio cholerae* is the *Vibrio cholerae* protease (PrtV). Using reverse molecular genetics techniques an extracellular protease was identified as PrtV protein as being necessary for killing nematodes by *Vibrio cholerae* [26]. The killing effect is associated with colonization of the *C. elegans* intestine. The nematode *Caenorhabditis elegans* has been used successfully as an invertebrate model to screen for virulence factors of several human pathogens e.g. *P. aeruginosa* and *S. typhimurium* [27, 28]. *V. cholerae* lethal
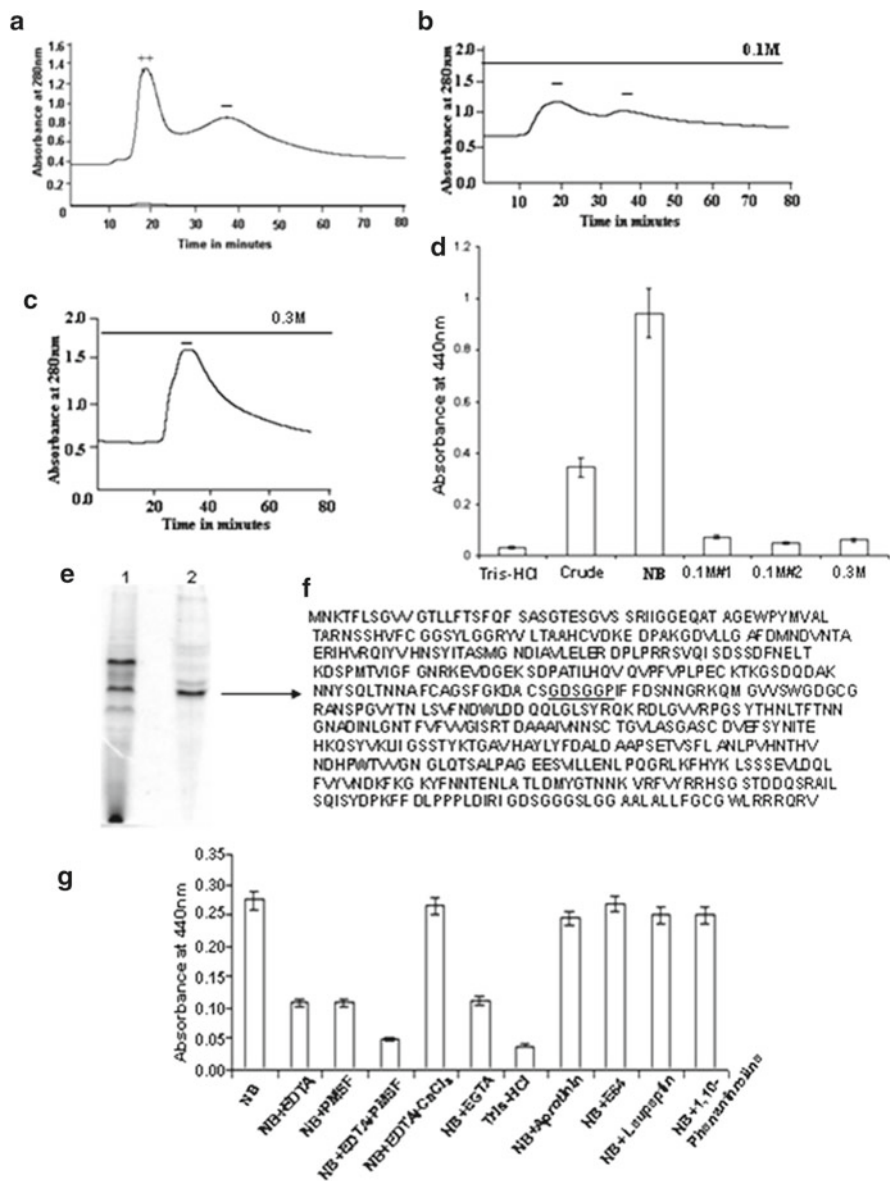
Fig. 4 Partial purification and identification of protease. Chromatographic profile of ammonium sulphate precipitated crude proteins from culture supernatants of CHA6.8Δ*prtV* strain loaded onto an anion exchange column (DE-52). (**a**) Proteins eluted in the non-binding fraction (NB), (**b**) proteins eluted with 0.1 M NaCl, (**c**) proteins eluted with 0.3 M NaCl, +/− shows presence or absence of protease activity, (**d**) azocasein assay with pooled samples (30 μg) NB, 0.1M#1, 0.1M#2, 0.3 M and crude proteins. (**e**) Native PAGE profile (*lane 1*) of crude proteins of CHA6.8Δ*prtV* strain and (*lane 2*) of partially purified protease (NB) from DE-52 column. The marked protein band was analyzed by MS/MS sequencing and the peptides *highlighted* showed homology with a 59-kDa trypsin-like serine protease encoded by VC1649. (**f**) The *underlined* GDSGGP are the amino acid sequences around the serine residue present in trypsin-like serine proteases. (**g**) Protease inhibition test of NB fraction (5 μg) with protease inhibitors 10 mM EDTA, 25 mM PMSF, 25 mM PMSF and 10 mM EDTA, 10 mM EDTA and 20 mM CaCl₂, 10 mM EGTA, 1 μg/ml aprotinin, 28 mM E64, 1 μg/ml leupeptin and 10 mM 1,10-phenanthroline incubated for 30 min at 37 °C. Residual protease activity was assayed by azocasein assay. Twenty-five mM Tris-HCl was used as a negative control. The values shown are the means with standard deviations from three experiments

infection of the *C. elegans* depends on a HapR-regulated protease. HAP encoded by *hapA* gene is regulated by *hapR*, and is the major extracellular protease. However, when *hap*A mutant *V. cholerae* was tested in *C. elegans* killing assay no attenuation was found in comparison with the wild type. The culture supernatant from *hapA* mutant contained some proteins not found in the supernatant of *hapR* mutant [26]. The three most abundant putative *hapR*-regulated proteins detected in the supernatants from the *hapA* mutant *V. cholerae* by mass spectrophotometer revealed that the protein bands corresponded to proteins encoded by the ORFs VCA0812, VCA0813 and VCA0223. The protein products are a leucine aminopeptidase-related protease, leucine amino aminopeptidase (Lap) [29] and the PrtV protease [30] respectively. Knock mutants Δ*prtV*, Δ*lap* and Δ*lapX* mutants were tested in *C. elegans* model and of all the mutants only Δ*prtV* mutant showed complete attenuation in comparison to wild type. Neither of the Δ*lap* and Δ*lapX* mutants showed significant attenuation. Furthermore, the cloned *prtV* gene resulted in an increased killing effect by the wild type *V. cholerae* strain C6706, and it restored a significant killing effect in the case of the *hapR* mutant derivative. PrtV is important for *V. cholerae* inhibition of natural Bacteriovorous predators. *V. cholerae* caused a marked reduction in the number of viable protozoa (*Cafeteria roenbergensis* and *Tetrahymena pyriformis*) during prolonged co-cultivation, whereas the bacterial density remained virtually unaltered. Furthermore *V. cholerae* with mutations abolishing the *hapR* or *prtV* genes appeared strongly attenuated in the predator grazing test, and most of the bacteria were consumed by the predator within 3 days. The metalloprotease PrtV was purified from the culture supernatants of a *Vibrio cholerae* derivative that is deficient in several other secreted peptidases, including the otherwise abundant hemagglutinin protease [31]. The PrtV is synthesized as a 102 kDa protein, but undergoes several N-and C-terminal processing steps during *V. cholerae* envelope translocation and prolonged incubation [31]. Purified *V. cholerae* PrtV protease forms of 81 or 73-kDa were stabilized by calcium ions. Removal of calcium resulted in further rapid autoproteolysis. In assay using cultured cells of the human intestinal cell line HCTS, the PrtV protein showed a cytotoxic effect leading to cell death. PrtV also degraded the extracellular matrix components fibronectin and fibrinogen. PrtV mutants were not attenuated in the infant mouse model, nor did they exhibit reduced colonization potential compared with wild type in competition experiments [30]. PrtV also modulates the response of *Vibrio cholerae* hemolysin in human intestinal epithelial cells. *Vibrio cholerae* hemolysin is capable of causing an inflammatory response characterized by increased permeability and production of IL-8 and TNF-alpha in tight monolayers. The inflammatory response was totally abolished by treatment with PrtV [32].

2.3   59-kDa Serine Protease

Although HAP is a very active virulence factor, an isogenic strain of *V. cholerae* mutated in the *hap* gene was no less virulent in infant rabbits than the parental strain [24].

An earlier study by Hase et al. [33] showed that a *hapA*-deleted mutant of *V. cholerae* O1 had reduced extracellular proteolytic activity compared with the parental strain in a skim milk assay, indicating that the mutant still produces some extracellular proteolytic activity. In addition, residual proteolytic activity expressed by the *hapA*-deleted mutant is distinct from HAP, as demonstrated by failure of anti-HAP serum to inhibit the activity of this secondary protease on milk agar. The mutant strain also failed to agglutinate chicken erythrocytes [24]. Young and Broadbent [34] described several extracellular proteases in *V. cholerae* that could explain the residual proteolytic activity of the *hap*-negative *V. cholerae* mutant. Besides HAP, the other major well-characterized protease in *V. cholerae* is a 97-kDa *Vibrio cholerae protease*, PrtV. PrtV plays a role in virulence in a *C. elegans* infection model [26].

A *hapA* and *prtV* double knock out mutant of *V. cholerae* strain CHA6.8Δ*prtV* still had residual protease activity [7]. This protease was partially purified from strain CHA6.8Δ*prtV*. The ammonium sulphate precipitated proteins from culture supernatants of CHA6.8Δ*prtV* were loaded onto an anion exchange chromatography column (DE-52) [7]. The proteins in the non-binding (NB) fraction of the column (Fig. 4a) were pooled and concentrated. The bound proteins were eluted with 0.1 M (Fig. 4b) and 0.3 M (Fig. 4c) NaCl, dialyzed against 25 mM Tris-HCl buffer and concentrated. When protease activity in the NB, 0.1 M and 0.3 M NaCl eluted fractions were tested by azocasein assay, the major protease activity was present in the NB fraction (Fig. 4d). The NB-pooled fraction was concentrated and run on a native PAGE (Fig. 4e). The major protein band was excised and analyzed by MS/MS sequencing (Fig. 4e). The sequences highlighted showed homology with a 59-kDa serine protease encoded by the gene VC1649 (Fig. 4f). The sequence GDSGGP (underlined) flanks the serine residue in trypsin-like serine proteases (Fig. 4f). To determine the nature of the partially purified protease from CHA6.8Δ*prtV* eluted in the non-binding fraction of a DE52 anion-exchange column, we performed protease inhibition assays with several inhibitors (Fig. 4g). The protease was partially inhibited in the presence of EDTA (60.3 %), EGTA (59.2 %) and PMSF (60.3 %). The partially purified protease was completely inhibited when PMSF and EDTA are used together (Fig. 4g). There was significantly less inhibition of protease activity in the presence of 1, 10-phenanthroline (9 %), aprotinin (10.5 %), leupeptin (8.7 %) and E64 (1.8 %). Although EDTA inhibited protease activity by 60.3 %, EDTA in the presence of $CaCl_2$ inhibited activity by only 3.6 % inhibition (Fig. 4g). The serine protease secreted byCHA6.8Δ*prtV* is a calcium-dependent serine protease.

To study the role of the 59-kDa serine protease in virulence, 50 μg of the partially purified protease was injected into the rabbit ileum, which induced significant hemorrhagic fluid accumulation (FA ratio 1.2+/20.2, n=3, Fig. 5a). When a similar concentration of the protease was incubated in the presence of PMSF and EDTA and injected into the rabbit ileum, there was a significant decrease in fluid accumulation (FA ratio 0.3+/20.05, n=3, Fig. 5a). Histopathological analysis of the rabbit ileum revealed that the protease caused extensive damage to all the layers of the mucosa. There was damage to the villus surface structure with hemorrhage in all layers of the mucosa. On the other hand, analysis of the ileal tissues treated with the
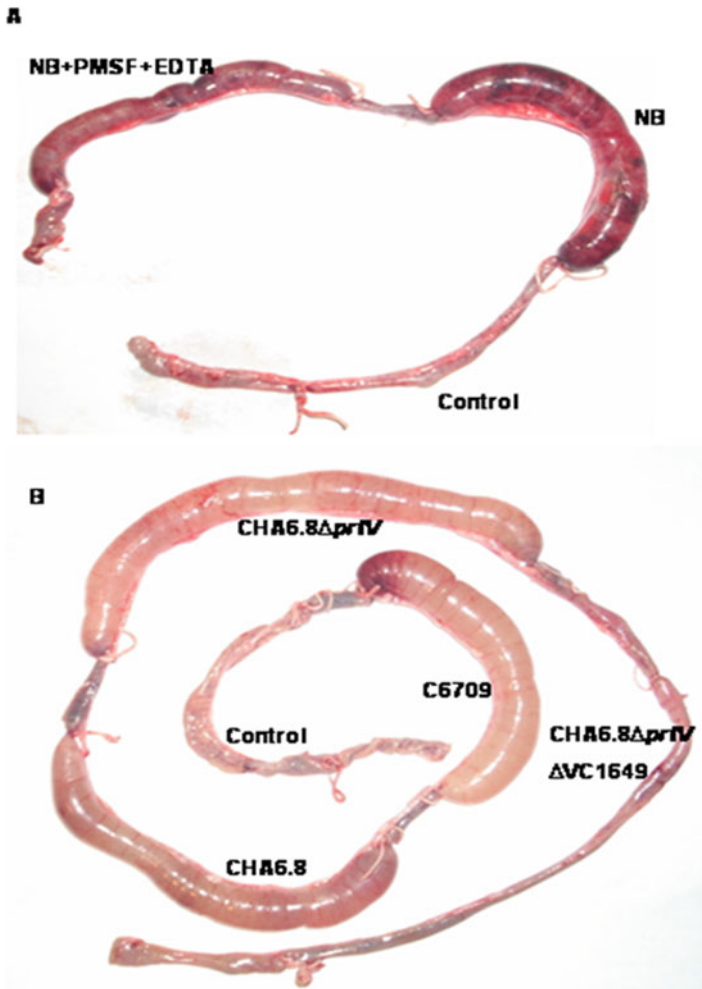
Fig. 5 Rabbit ileal loop assay. (**a**) RIL response of partially purified protease (50 μg, NB) showing significant hemorrhagic fluid accumulation (FA ratio 1.2 +/−0.2 n = 3) and its effect after inhibition with 25 mM PMSF and 10 mM EDTA (NB + PMSF + EDTA) shows significant decrease in fluid accumulation (FA ratio 0.3 +/−0.05 n = 3). Twenty five mM Tris-HCl with 25 mM PMSF + 10 mM EDTA was used as a negative control (FA ratio = 0.12 +/−0.002, n = 3). (**b**) RIL response with culture supernatants of C6709 (FA ratio 1.1 +/− 0.3, n = 3), CHA6.8 (FA ratio 1.08 +/− 0.2, n = 3), CHA6.8Δ*prtV* (FA ratio 1.02 +/− 0.2, n = 3), CHA6.8Δ*prtV*ΔVC1649 (FA ratio 0.11 +/− 0.005, n = 3) and Tryptic soy broth as negative control (FA ratio 0.09 +/− 0.002, n = 3)

protease in presence of both PMSF and EDTA, revealed normal microvillus structure with no gross alteration in villus structure, although the villus lamina propria was slightly dilated and RBCs had accumulated in a few places in the basal area. PMSF and EDTA completely inhibited protease activity (Fig. 5a), but still we observed some residual effect in the rabbit ileal loop. This effect could be due to some other domain in the protease, which may not be its proteolytic domain, and

could be responsible for causing damage to the ileal tissue. Tissues treated with 25 mM Tris-HCl and PMSF+EDTA did not cause fluid accumulation in RIL (FA ratio 0.12+/20.002 n=3, Fig. 5a)

Rabbit ileal loop assay with one ml of culture supernatant of C6709, CHA6.8 and CHA6.8Δ*prtV* strain induced significant fluid accumulation (Fig. 5b) in RIL model (FA ratio 1.1+/20.3, n=3; 1.08+/20.2, n=3; and 1.02+/20.2, n=3 respectively) where as CHA6.8ΔprtVΔVC1649 strain which is devoid of the serine protease gene and sterile tryptic soy broth, TSB (negative control) did not induce any fluid accumulation (Fig. 5b) (FA ratio 0.11+/20.005, n=3 and 0.09+/20.002, n=3 respectively). Almost similar results were observed when one ml of washed bacterial cells (10^9 cfu/ml) of C6709, CHA6.8 and CHA6.8Δ*prtV* induced significant fluid accumulation (FA ratio 1.2+/20.35, n=3; 1.1+/20.3, n=3; and 1.0+/20.2, n=3 respectively). Bacterial cells of CHA6.8ΔprtVΔ*VC1649* strain did not induce any fluid accumulation (FA ratio 0.15+/20.005, n=3). Ileal tissues treated with culture supernatant of CHA6.8Δ*prtV* strain also showed dilated villi with gross hemorrhage in all layers of the mucosa. The ileal tissues treated with culture supernatant of CHA6.8Δ*prtV*Δ*VC1649* strain protease showed villous architecture almost normal with minimum hemorrhage in mucosa and sub-mucosa.

V. vulnificus also secretes a 59-kDa serine protease, which is the free form, while the 69-kDa protein may be a complex form associated non-covalently with small peptide(s) [35]. The proteolytic activity of the final preparation was almost completely abolished by treatment with 5 mM PMSF, a well-known inhibitor of serine proteases. By contrast, tetraethylenepentamine, a specific inhibitor of metalloproteases including VVP (*Vibrio vulnificus* protease), showed no inhibitory effect on the proteolytic activity. *Vibrio vulnificus* strain NCIMB 2137, in which *vvp* has been deleted, secretes a serine protease, VVA0302. *V. vulnificus* serine protease may be a virulence factor in vibriosis, which is characterized by external and internal hemorrhages affecting the major organs [36], or human wound infection with necrotic tissue damage [37].

The proteomic analysis of the *V. cholerae* type II secretome revealed the presence of three related serine proteases named as (ves: vibrio extracellular serine proteases) *vesA*, *vesB* and *vesC* [38]. Experiments suggest that *vesA*, *vesB* and *vesC* do not contribute to the intestinal survival and colonization capability of *V. cholerae* in mice. VesA has shown to be responsible for processing the A subunit of cholera toxin during in-vitro growth of *V. cholerae* strain N 16961. *VesC* encoded by VC1649 (59-kDa serine protease) is not capable of processing cholera toxin under tested conditions but has been shown by us to play a role in hemorrhagic response in rabbit ileal loop and it may play a role in pathogenesis [7].

3   Leucine Aminopeptidase

The aminopeptidases (APs) form a group of zinc-dependent metalloproteases that catalyze the removal of amino acids from the N terminus of a protein. These enzymes are of biological and medical importance because of their role in protein

degradation and in the metabolism of biologically active peptides. Leucine APs (LAPs) preferably cleave leucyl substrates, although substantial rates of enzymatic cleavage with most amino-terminal residues are observed [39]. The structural gene for the *Vibrio cholerae* leucine aminopeptidase (lap) has been cloned and sequenced [29]. The molecular weight of LAP is 54.4 kDa. The deduced amino acid sequence of the entire protein showed high homology with the sequence of *Vibrio proteolyticus* leucine aminopeptidase. The protease showed maximum activity at pH 9.0 and was thermostable at 70 °C. The substrate leucyl-p-nitroanilide was cleaved by the protease, and its activity was inhibited by EDTA and bestatin. LAP may play an important role in releasing the free amino acids needed for the regulation of expression of other virulence factors [40].

4   Conclusions

V. cholerae, secretes several proteases like HAP, PrtV and 59-kDa serine protease. The major protease secreted by *V. cholerae* strains is the HAP. Almost all results suggest indirect pathogenic roles of HAP, the possibility of a direct role is also reported. Ghosh et al. [6] assayed the enterotoxic activity of HAP purified from a cholera toxin-negative *V. cholerae* strain. The purified HAP showed a positive hemorrhagic fluid response in the rabbit ileal loop assay and an increase in the intestinal short circuit current in the Ussing chamber assay. A metalloprotease, other than HAP, PrtV is thought to play a role in aquatic environment. Vaitkevicius et al. [26] showed the effect of PrtV against predator grazing in aquatic environment. PrtV also plays a role in pathogenicity of *V. cholerae*. The serine protease from a Δ*hapA*Δ*prtV V. cholerae* O1 strain induced hemorrhagic response in rabbit ileal loop [7]. Role of proteases in pathogenicity in *V. cholerae* could also depend on the expression levels of proteases. In an earlier study by Young and Broadbent [34], 100 strains of *V. cholerae* El Tor from different parts of the world were screened for protease production by a rapid assay with gelatin agar plates. Based on protease production, the strains were classified as high, medium and low protease producers. The expression of these proteases may play a role in pathogenicity of *V. cholerae* strains. Our results suggest that the expression of proteases in *V. cholerae* may follow a cascade of events. HAP controls secretion of the 59-kDa serine protease, which in turn may control secretion of other proteases.

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