

Expression, purification, and characterization of thermolabile hemolysin (TLH) from *Vibrio alginolyticus*

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ABSTRACT: Hemolysin is a putative pathogenicity factor in many bacterial pathogens. In this study, a DNA fragment containing the open reading frame (1254 bp) of the thermolabile hemolysin gene (*tlh*) from *Vibrio alginolyticus* V05 was amplified and cloned into the expression plasmid pET-24d(+). The deduced amino acid sequence of the thermolabile hemolysin (TLH) shared 94 and 83% identity with the lecithin-dependent hemolysin (LDH)/TLH of *V. parahaemolyticus* and *V. harveyi* thermolabile hemolysin (VHH), respectively. The sequence analysis also indicated that it contained a GDSL lipase domain like VHH. The recombinant protein with a predicted molecular mass of 47.2 kDa was expressed in the *Escherichia coli* strain BL21 (DE3) as a His-tag fused protein. TLH purified by the nickel-nitrilotriacetic acid (Ni-NTA) His-Bind Resin method showed phospholipase activity on an egg yolk emulsion plate and hemolytic activity against flounder erythrocytes with a specific activity of 18 hemolytic units μg^{-1} . The addition of divalent cations at different concentrations decreased hemolytic activity of the purified TLH, but monovalent cations did not affect hemolytic activity. The hemolytic activity of TLH was also markedly inhibited by protein modification reagents, i.e. β -mercaptoethanol, phenylmethylsulfonyl fluoride, and 5,5'-dithio-bis(2-nitrobenzoic acid). Moreover, TLH was toxic to zebrafish when injected intraperitoneally, with a median lethal dose (LD_{50}) of 0.8 μg protein g^{-1} fish. This work shows that TLH could potentially be developed as a vaccine and used as a diagnostic tool for vibriosis.

KEY WORDS: *Vibrio alginolyticus* · Hemolysin · Expression · Purification · Pathogenicity

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INTRODUCTION

Vibriosis is a systemic disease of marine fish that is characterized by general bacterial septicemia, hemorrhaging, and skin lesions. The causative agents of vibriosis are a group of bacterial pathogens belonging to the genus *Vibrio* (Egidius 1987, Zhang & Austin 2005, Jayaprakash et al. 2006). *V. alginolyticus* is a gram-negative, estuarine bacterial species and is the closest relative of *V. parahaemolyticus* (González-Escalona et al. 2006, Cai et al. 2007). *V. alginolyticus* has been described as an important pathogen, and is responsible for the ulcer disease of gilthead sea bream *Sparus aurata*, turbot *Scophthalmus maximus*, crimson snapper *Lutjanus erythropterus*, sea mullet *Mugil cephalus*, and giant freshwater prawn *Macrobrachium rosenbergii* (Kim et al. 2000, Selvin & Lipton 2003, Jaya-

prakash et al. 2006, Cai et al. 2007). Previous studies have shown that *V. alginolyticus* is a major infective pathogen for silver sea bream *Sparus sarba*, an edible fish of major economic value to Hong Kong (Li et al. 2003). Enzymatic characterization of the isolated extracellular products (ECP) of pathogenic *V. alginolyticus* demonstrated the presence of hemolysin that was specific only to fish blood (Li et al. 2003).

Hemolysin, an exotoxin that lyses erythrocyte membranes with the liberation of hemoglobin, is arguably the most widely distributed toxin among pathogenic *Vibrio* spp. and exerts various roles in the infection process (Shinoda 1999). There are 5 representative hemolysin families in *Vibrio* spp., including the thermostable direct hemolysin (TDH) family, the HlyA (or E1 Tor hemolysin) family, the thermolabile hemolysin (TLH) family, the thermostable hemolysin (δ -VPH) family, and a

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novel hemolysis gene (HLX) family (Zhang & Austin 2005). TDH and HlyA have been studied extensively and are closely associated with virulence (Zhang & Austin 2005, Cai et al. 2007). However, the role of some other hemolysins, e.g. TLH, δ -VPH, and HLX, is unclear and will need to be determined by further research (Singh et al. 2009), although previous studies have indicated that TLH is widespread among *Vibrio* (Wang et al. 2007, Parvathi et al. 2009). In *V. alginolyticus*, the hemolysins of TDH and thermostable direct hemolysin-related hemolysin (TRH) have been well studied (González-Escalona et al. 2006, Cai et al. 2007), but the TLH hemolysin of this bacterium has not been characterized yet.

In the present study, we cloned and expressed the *tlh* gene from *Vibrio alginolyticus* in *Escherichia coli*, purified the recombinant TLH, and investigated some of its biological and biochemical characteristics.

MATERIALS AND METHODS

Bacterial strains, plasmids, and test animals. *Vibrio alginolyticus* V05 and *V. harveyi* V01 that had previously been isolated from infected sea bream cultured in Tolo Harbour, Hong Kong, were supplied by the Biology Department, the Chinese University of Hong Kong. *V. alginolyticus* VIB 283, and *V. harveyi* VIB 645 and VIB 647 were obtained from the School of Life Sciences, Heriot-Watt University, UK. These strains were cultured at 28°C on marine 2216E agar plates. The expression vector pET-24d (+) (with a C-terminal His-Tag) and the *E. coli* strain BL21 (DE3), used for overexpression of TLH, were purchased from Novagen. Luria-Bertani (LB) medium was used for the routine culturing of *E. coli* at 37°C. The *E. coli* transformants with recombinant plasmid were maintained on LB agar containing kanamycin (LB-Kan, 50 $\mu\text{g ml}^{-1}$).

Zebrafish *Danio rerio* were purchased from the market in Qingdao, and the fish were maintained in an aquarium at 26 to 30°C during the experiments involving lethality tests of TLH.

DNA extraction. Total bacterial genomic DNA from *Vibrio alginolyticus* and *V. harveyi* strains were prepared using the TIANamp Bacteria DNA Kit (Tiangen) according to the manufacturer's instructions.

Construction of an overexpression vector. The *tlh* gene was obtained by PCR amplification using the DNA of *Vibrio alginolyticus* V05 as template. According to the *ldh/tlh* hemolysin gene (1254 bp) of the whole genome shotgun sequence from *V. alginolyticus* 12G01 (GenBank accession no. NZ_AAPS01000004), a specific primer set (TLHF and TLHR) was designed. The forward primer (TLHF: 5'-CCG GAA TTC ATG AAA AAA ACA ATC ACA CT-3') begins from the initiation codon

and adds an *EcoRI* site at the 5' end of the gene, and the reverse primer (TLHR: 5'-CGC TCG AGA AAG CGA AAC TCT TCT AAG T-3') ends before the stop codon, and adds an *XhoI* site. The primer set was tested in PCR with DNA templates prepared from different strains of *V. alginolyticus* (V05, VIB283) and *V. harveyi* (V01, VIB645, VIB647), using 50 ng DNA template, 1 \times PCR buffer, 1.5 mM of MgCl_2 , 200 μM of each dNTP, 0.5 μM concentration of each primer, and 0.5 U of *Taq* DNA polymerase (Fermentas) in a total volume of 20 μl . The PCR conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 50°C for 40 s, and 72°C for 1.5 min, and a final extension of 10 min at 72°C. The PCR product of *V. alginolyticus* V05 (1268 bp) was excised and inserted into the *EcoRI/XhoI*-cut expression vector pET-24d (+). The ligated plasmid was transformed into *E. coli* BL21 (DE3) for expression of the full-length *tlh* gene. The sequence of the cloned gene in the pET-24d (+) vector was confirmed by DNA sequencing (Biosung).

Expression and purification of TLH from transformed *E. coli*. The expression of TLH was performed as described by Zhong et al. (2006). In brief, a single colony of *E. coli* BL21 (DE3) transformed with pET-24d (+)/*tlh* was inoculated into 5 ml of LB-Kan broth and incubated at 37°C for 12 h with shaking at 180 rpm. This culture was inoculated into 495 ml of the same medium and allowed to grow for another 2.5 h, until the optical density (OD_{600}) value reached 0.6. Expression of TLH was induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, with additional incubation at 25°C for 6 h.

To purify the TLH from the bacterial cells, the induced cells were harvested by centrifugation at 4000 $\times g$ for 20 min at 4°C, and the pellet was resuspended in 50 ml nickel-nitrilotriacetic acid (Ni-NTA) bind buffer containing 10 mM imidazole, and then lysed by ultrasonication and centrifuged at 10 000 $\times g$ for 30 min at 4°C. The supernatant was checked for expression and processed for purification. Purification of poly His-tagged TLH was performed using Ni-NTA His-Bind Resin according to the manufacturer's instructions (Novagen). The TLH was purified by performing a 2-step elution with 50 mM imidazole after the 20 mM imidazole wash, followed by 250 mM imidazole elution. The expression and purity of TLH were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration of TLH was determined by the dye-binding method of Bradford (1976). For signal sequence prediction, the SignalP program (www.cbs.dtu.dk/services/SignalP-3.0) was used (Nielsen et al. 1999).

Assay of hemolytic activity and phospholipase activity. Hemolytic activity against flounder *Paralichthys olivaceus* erythrocytes was measured by a

previously published procedure (Zhang et al. 2001). In brief, the purified TLH (100 hemolytic units, HU) was added to an Oxford cup (5 mm diameter), which was placed on the plate and incubated at 37°C for 8 h. The hemolytic activity against sheep erythrocytes was measured as above except that it was performed on Columbia blood agar base with sheep erythrocytes (HopeBio), supplied with TLH of 100, 200, 500, 1000, 3000, 4000, and 6000 HU, which were reacted for 10 h at 37°C. A hemolytic titer was carried out in a 96-well microtiter plate (Costar), in which 100 µl TLH were diluted 2-fold in 20 mM Tris-buffered saline (TBS, pH 7.5) and mixed with equal volumes of the 2% (v/v) erythrocyte suspension. TBS was used as negative control. The plate was incubated at 37°C for 2 h, and the hemolytic titer was recorded as the reciprocal of the dilution that gave an end product of 50% hemolysis, and that reciprocal of the dilution was defined as 1 HU. All the hemolytic activity experiments were performed in triplicate.

Phospholipase activity was detected as previously described (Montero & Austin 1999, Zhong et al. 2006). A 20 µl aliquot of TLH (100 HU) was added to Oxford cups, which were placed on 1% (v/v) egg yolk emulsion and incubated at 28°C for 24 h. The lipase domain in the deduced amino acid sequence was analyzed via Pfam (Wellcome Trust Sanger Institute: <http://pfam.sanger.ac.uk/>).

Effects of metallic cations on hemolytic activity. The effect of cations on hemolysis was determined by addition of various concentrations of metallic cations (Na⁺, K⁺, Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, and Ba²⁺) as the chloride forms in 20 mM TBS (pH 7.5; Jeon et al. 2009). The metallic cation solution was added to TLH (100 HU), and the mixture was incubated at 30°C for 30 min and immediately subjected to the hemolytic assay. The hemolytic activity experiment was performed in a 96-well microtiter plate as described above. In brief, the mixtures (100 µl) were double diluted and mixed with equal volumes of the 2% (v/v) erythrocyte suspension. The residual hemolytic activity was recorded. The mixtures of each metallic cation and erythrocyte were used as controls in doubling dilutions.

Effects of modification reagents on hemolytic activity. To determine functional groups in the TLH structure, TLH (100 HU) was mixed with various concentrations (0, 0.1, 1, 10 mM) of chemical modification reagents and incubated at 30°C for 30 min. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), 2,4,6-trinitrobenzene sulfonate (TNBS), diethyl pyrocarbonate (DEPC), β-mercaptoethanol (βME), phenylmethylsulfonyl fluoride (PMSF), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were used to modify the carboxy group in glutamic acid/aspartic acid, amino group, imidazolyl in histidine, disulfide

bond, hydroxy group in serine, and hydrosulfide group in cysteine, respectively (Kazan & Erarslan 2001, Sultan et al. 2004). The hemolytic activity of the mixtures was detected as described.

Fish pathogenicity. Zebrafish from quarantined stocks recognized as disease-free (Austin & Austin 1989) were used as models to assess pathogenicity as described below (Zhong et al. 2006). Groups of 10 fish (average weight: 0.447 g) were infected by intraperitoneal injection with 25 µl volumes of 2-fold dilutions of TLH preparations. Sterile TBS was also injected as parallel controls. Mortality of fish was recorded for 7 d after injection. The median lethal dose (LD₅₀) was calculated using the probit method described by Wardlaw (1985).

RESULTS

Construction of an overexpression vector

The full-length sequence of *tlh* revealed an open reading frame (ORF) of 1254 bp that encodes a 417 amino acid polypeptide with a predicted molecular mass of 47.2 kDa and an isoelectric point (pI) of 5.08 (GenBank accession no. GQ300939). The deduced amino acids of the TLH showed 94 and 83% homology to the lecithin-dependent hemolysin (LDH)/TLH of *Vibrio parahaemolyticus* (GenBank accession no. EDM60410) and *V. harveyi* thermolabile hemolysin (VHH; GenBank accession no. AF293430), respectively. The PCR primers TLHF and TLHR amplified a ~1.3 kb DNA fragment containing the full-length *tlh* gene from *V. alginolyticus* strains, but not in a PCR using DNA templates from strains of *V. harveyi* (Fig. 1). Amplified product from V05 (Fig. 1, Lane 1)

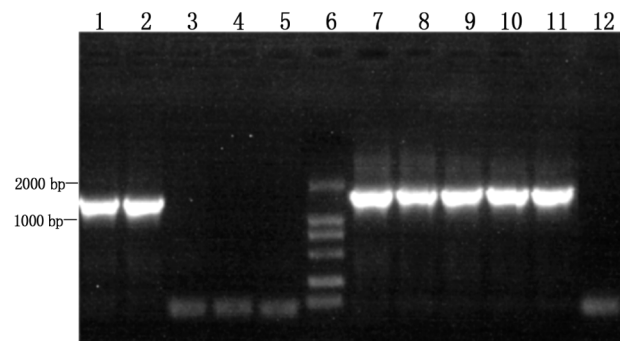


Fig. 1. Results of the PCR using different DNA as templates. Lanes 1–5: amplification of the hemolysin gene using primers TLHF and TLHR, the PCR template from *Vibrio alginolyticus* V05, *V. alginolyticus* VIB 283, *V. harveyi* VIB645, *V. harveyi* VIB647, and *V. harveyi* V01, respectively; Lane 6: marker DL2000; Lanes 7–11: amplification of the 16srDNA as positive control, using the above strains in the same order; Lane 12: negative control (no template)

was cloned into *E. coli* BL21 (DE3) using the pET-24d (+) vector. A stop codon was not incorporated in the reverse primer so that a histidine tag would be expressed at the C-terminus of TLH to simplify subsequent purification procedures.

Purification of TLH from transformed *E. coli*

TLH was found to exist in both the culture supernatant and bacterial cells, but the yield of TLH from the culture supernatant was lower than that obtained from the bacterial cells. Intracellular TLH was purified by Ni-NTA His-Bind Resin, and 5 ml protein (2.199 mg ml^{-1}) were obtained. Based on the analysis of SignalP, a leader peptide of 20 amino acids should be removed from the fused TLH (48.2 kDa) containing poly His-tag to form a mature peptide with a predicted molecular mass of 46.2 kDa. SDS-PAGE analysis of the purified TLH from transformed cells revealed a single band with an estimated molecular weight of ~45 kDa (Fig. 2).

Hemolytic and phospholipase activities

The culture of induced *E. coli* with recombinant plasmid displayed hemolytic and phospholipase activities compared to non-induced transformed cells (Figs. 3 & 4). The appearance of a lytic zone on blood agar and the presence of an opalescent zone around the well on egg yolk emulsion plates were recorded as evidence of positivity. The sheep erythrocytes were not very sensi-

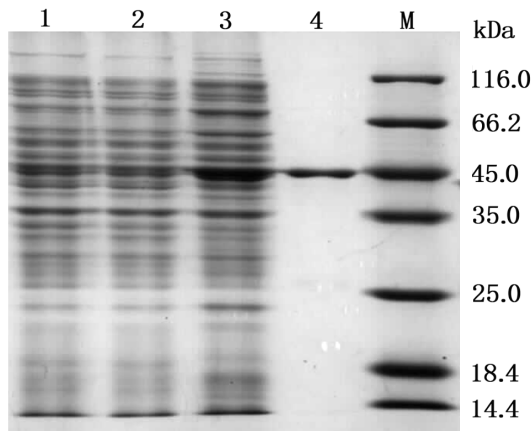


Fig. 2. Expression of *tlh* in the pET-24d (+) vector using *Escherichia coli* BL21 (DE3) as the host. Lane 1: negative control without the *tlh* fragment; Lane 2: uninduced cell pellet fraction; Lane 3: induced cell pellet fraction; Lane 4: purified *Vibrio alginolyticus* thermolabile hemolysin from transformed *E. coli* cells; Lane M: molecular marker

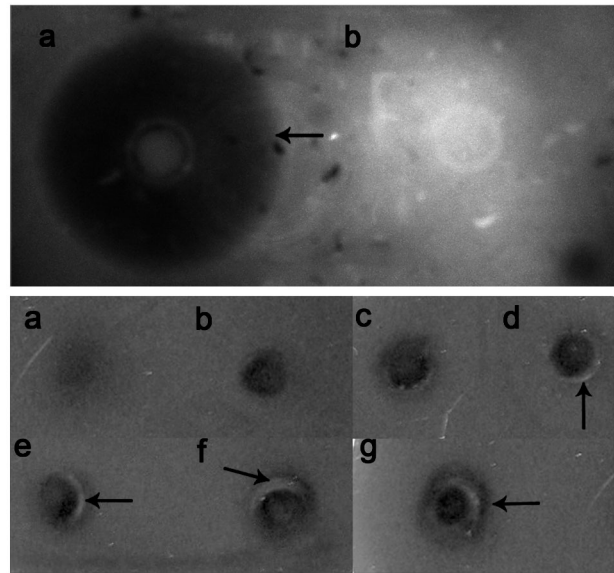


Fig. 3. Hemolytic activity of *Vibrio alginolyticus* thermolabile hemolysin (TLH) on blood agar plate. Top: TLH on a flounder blood agar plate. (a) purified TLH from transformed *Escherichia coli* cells; (b) whole cell lysates of uninduced sample. Bottom: TLH on a sheep blood agar plate. Purified TLH of 100, 200, 500, 1000, 3000, 4000, and 6000 hemolytic units (HU) was added to Oxford cups from (a) to (g), respectively. Arrows show the sites of hemolysis

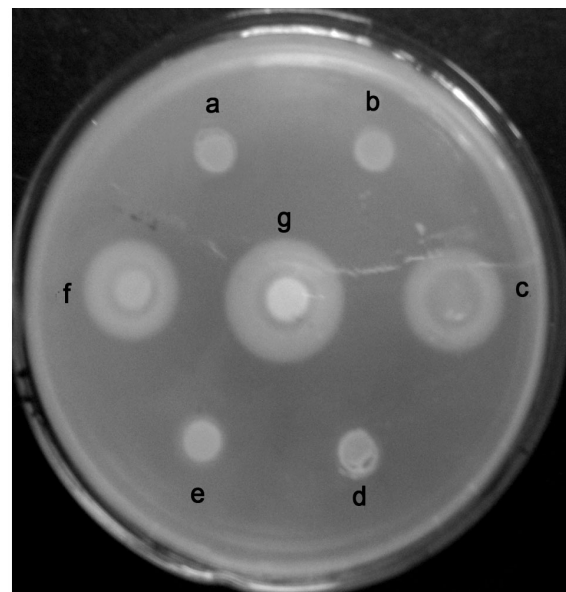


Fig. 4. Phospholipase activity of *Escherichia coli* BL21 (DE3) containing recombinant plasmid and purified thermolabile hemolysin (TLH) was analyzed on an egg yolk emulsion plate. Culture supernatant of the (a) negative control without *tlh*, (b) uninduced sample, (c) induced sample. Whole cell lysates of the (d) negative control without *tlh*, (e) uninduced sample, (f) induced sample. (g) Purified TLH from transformed *E. coli* cells

tive to TLH hemolysin, and the TLH could display obvious hemolytic activity up to 6000 HU (compared to 100 HU against flounder erythrocytes). The specific activity of the purified TLH was 18 HU μg^{-1} , as calculated using a hemolytic titer. Based on the Pfam analysis, the predicted protein had one GDSL-like lipase/acylhydrolase domain from 146 to 405 amino acids.

Effects of metallic cations on hemolytic activity

As shown in Table 1, Na^+ and K^+ did not affect hemolytic activity at 1 mM and 25 mM concentrations. At 1 mM concentration, Mg^{2+} , Ca^{2+} , Mn^{2+} , and Ba^{2+} decreased the activity to 25%, and Co^{2+} , Ni^{2+} , and Zn^{2+} decreased the activity by 50%. However, addition of Cu^{2+} at 1 mM caused the erythrocytes to rupture. The addition of all divalent cations decreased the activity by 50% at 0.1 mM. When the concentration of Zn^{2+} was increased to 25 mM, the hemolytic activity was decreased to 25%. No metallic cations, except Cu^{2+} at 1 mM, caused the destruction of erythrocytes by themselves.

Effects of modification reagents on hemolytic activity

As shown in Table 2, the effect of 6 chemical modification reagents on hemolytic activity was detected at 0, 0.1, 1, and 10 mM. TNBS and DEPC decreased the activity by 50% at 0.1, 1, and 10 mM concentrations. The other 4 reagents also decreased the activity by 50% at 0.1 mM, and EDC·HCl at 1 mM caused the hemolytic activity to remain at 50%. When the concentration of βME , PMSF, and DTNB was increased to

Table 1. Effects of metallic cations on the hemolytic activity of *Vibrio alginolyticus* thermolabile hemolysin. Metallic cations were dissolved in Tris-buffered saline. ND: not determined

Metallic cation	Relative hemolytic activity (%)		
	0.1 mM	1 mM	25 mM
Na^+	ND	100	100
K^+	ND	100	100
Mg^{2+}	50	25	ND
Ca^{2+}	50	25	ND
Mn^{2+}	50	25	ND
Co^{2+}	50	50	ND
Ni^{2+}	50	50	ND
Cu^{2+}	50	ND	ND
Zn^{2+}	50	50	25
Ba^{2+}	50	25	ND
None	100	100	100

Table 2. Effect of chemical modification reagents on the hemolytic activity of *Vibrio alginolyticus* thermolabile hemolysin. EDC·HCl: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; TNBS: 2,4,6-trinitrobenzene sulfonate; DEPC: diethyl pyrocarbonate; βME : β -mercaptoethanol; PMSF: phenylmethylsulfonyl fluoride; DTNB: 5,5'-dithiobis (2-nitrobenzoic acid)

Modification reagent	Relative hemolytic activity (%)		
	0.1 mM	1 mM	10 mM
EDC·HCl	50	50	25
TNBS	50	50	50
DEPC	50	50	50
βME	50	25	12.5
PMSF	50	25	12.5
DTNB	50	12.5	6.25
None	100	100	100

1 mM, the hemolytic activity was decreased to 25, 25, and 12.5%, respectively. There was continued decline in hemolytic activity, with losses of 75, 87.5, 87.5, and 93.75% of activity after the addition of EDC·HCl, βME , PMSF, and DTNB at 10 mM concentration, respectively. Moreover, the modification reagents did not rupture the erythrocytes by themselves at concentrations of 0.1, 1, and 10 mM.

Pathogenicity of TLH to zebrafish

Mortalities among zebrafish occurred between 1 and 120 h after injection of TLH. Most infected fish exhibited hemorrhaging in the peritoneal cavity, and signs of hemorrhage and necrosis at the site of the injection. The 32-fold diluted TLH at a concentration of 0.069 μg μl^{-1} killed all fish when 25 μl volumes were injected. In comparison, the 64-, 128-, and 256-fold diluted protein killed 80, 70, and 10% of the fish, respectively (Table 3). Thus, the LD_{50} value of TLH to zebrafish was established as 0.8 μg protein g^{-1} fish.

Table 3. Pathogenicity of thermolabile hemolysin (TLH) to zebrafish *Danio rerio*. Groups of 10 fish (average weight: 0.447 g) were injected with 25 μl volumes of 2-fold dilutions of TLH preparations. Controls were injected with 25 μl volumes of Tris-buffered saline

Dose (μg protein g^{-1} fish)	Total mortality (n = 10)	Time to death (h)
0	0	–
0.231	0	–
0.463	1	120
0.925	7	4–104
1.850	8	2–96
3.700	10	1–9

DISCUSSION

Our understanding of the role of hemolysin in the pathogenesis of vibriosis has largely been based on the results of previous studies on purification, characterization, mutation, and pathogenicity of VHH (Zhang et al. 2001, Zhong et al. 2006, Sun et al. 2007). In this study, we cloned, overexpressed, and purified the *tlh* gene from *Vibrio alginolyticus*. The predicted molecular mass of fused TLH was 48.2 kDa, which was 2 to 3 kDa larger than that determined by SDS-PAGE. The gel mobility of proteins often reflects their physicochemical properties and posttranslational modifications beyond their molecular masses, which may cause inconsistencies between the mobility and molecular mass (Shirai et al. 2008). The other reason for the difference in size may be due to splicing of the signal peptide of TLH. Zhong et al. (2006) demonstrated that 20 N-terminal amino acids were deleted from the recombinated VHH when secreted into the medium. Another study also found that *V. fluvialis* hemolysin was secreted into the extracellular environment as a 79 kDa protein after cleavage of 25 residues in the N-terminal region (Han et al. 2002). The purified TLH had strong phospholipase activity on the egg yolk emulsion. It was reasoned that the TLH protein may well be a phospholipase just like the VHH hemolysin of *V. harveyi* (Zhong et al. 2006, Sun et al. 2007). Also, a GDSL lipase domain was found in the deduced amino acid sequence of TLH. It has been reported that some *Vibrio* hemolysins demonstrate phospholipase activity. For example, the lecithin-dependent hemolysin of *V. parahaemolyticus* has phospholipase A₂/lysophospholipase activity (Shinoda et al. 1991), and the hemolysin of *V. cholerae* O139 has phospholipase C activity (Pal et al. 1997). In addition, some *Vibrio* phospholipases show hemolytic activity on erythrocytes. For example, PhlA from *V. mimicus* demonstrated high hemolytic activity to rainbow trout and tilapia erythrocytes (Lee et al. 2002), and *V. vulnificus* extracellular phospholipase A₂ and lysophospholipase had hemolytic activity to sheep and mouse erythrocytes (Testa et al. 1984). In our study, regarding the effect of metallic cations on hemolytic activity, a consistent change was detected using either the hemolytic microtiter or egg yolk plate method (data obtained using the second method were not shown). These data revealed a correlation between hemolysin and phospholipase.

The hemolytic activity of several *Vibrio* hemolysins has been well documented to be inhibited by divalent cations, such as Ca²⁺, Mg²⁺, and Mn²⁺ (Miyake et al. 1989, Zitzer et al. 1995, Miyoshi et al. 1997, Han et al. 2002). Miyoshi et al. (1997) reported that the hemolytic activity of *V. mimicus* hemolysin was inhibited by addi-

tion of <1 mM Cu²⁺, Zn²⁺, or Ni²⁺, and reasoned that these cations may function as osmotic protectants against the increase in intracellular osmotic pressure arising from blockage of influx of extracellular water via the toxin-induced pore at the cell membrane (Miyoshi et al. 1997). Hemolysins from *V. metschnikovii*, *V. cholerae* E1 Tor, and *V. fluvialis* were also influenced by divalent cations (Miyake et al. 1989, Zitzer et al. 1995, Han et al. 2002). Thus, it is relevant that the hemolytic activity of TLH was decreased to 50 % by addition of all the divalent cations at 0.1 mM concentration, i.e. Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, and Ba²⁺, and that the hemolytic activity was not affected by addition of monovalent cations (Na⁺ and K⁺). However, the mechanism by which these cations inhibit the hemolytic reaction is poorly understood. The hemolytic activity is possibly decreased by the potent oxidation of divalent cations, and the side-chain radical or the ionic environment of TLH may also take effect in the reaction.

Chemical modification of proteins is a convenient method for the investigation of the structure and function of enzymes and the composition of the enzyme active center (Zhou & Wang 1988). Our results showed that TLH was sensitive to the modifications of the hydroxy group and the disulfide bond, and especially sensitive to the hydrosulfide modification. These results indicated that there might be a serine residue containing a hydroxy group within the active site of TLH, and one or more disulfide bonds were necessary for the stable conformation. The hydrosulfide of cysteine might play an important role in the catalysis of TLH. Concurrently, the mutation of Ser153 on the conserved active Gly-Asp-Ser-Leu motif of VHH from *Vibrio harveyi* would result in the loss of all hemolytic activity (Sun et al. 2007).

TLH was lethal to zebrafish when injected intraperitoneally, and the LD₅₀ value of 0.8 µg protein g⁻¹ fish was similar to that of VHH in flounder, i.e. 1.2 µg protein g⁻¹ fish (Zhong et al. 2006). Comparatively, the lethal toxicity test of TDH from *Vibrio alginolyticus* showed that LD₅₀ values were 5.68 and 8.34 µg TDH g⁻¹ body weight for mouse and crimson snapper, respectively (Cai et al. 2007). The present demonstration of the toxicity of TLH on fish will facilitate the identification of the virulence factor that could be used as a candidate antigen for vaccine development and as a diagnostic tool for vibriosis.

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