Protease and Virulence of the Extracellular Products Produced by *Vibrio carchariae* after Growth on Various Media

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Protease and virulence of the extracellular products (ECP) of *Vibrio carchariae* strain EmI82KL, a causative agent of gastroenteritis in *Epinephelus coioides*, cultured on different media were studied. The bacteria grown on peptone agar, nutrient agar or brain heart infusion agar produced higher protease activities than that grown on tryptic soy agar (TSA) in terms of protein content. The addition of ethylenediamine di(o-hydroxyphenylacetic acid) or horse serum in TSA enhanced the ECP protease production while the addition of grouper serum apparently reduced the enzyme activity indicating the presence of protease inhibitor(s) in the fish serum. Furthermore, the use of grouper meat or peptone as a single nutrient source remarkably enhanced the production of ECP protease. Adding proteinaceous materials from animal sources (horse serum, grouper meat or peptone) on agar manifested higher ECP protease activity than that from plant source (TSA), indicating the intestine of carnivorous groupers might favour the existence, survival or infection of the bacterium. The protease was a metal-chelator-sensitive serine protease since it was inhibited by 3,4-dichloroisocoumarin and phenylmethanesulfonyl fluoride while about 80% of its activity inhibited by chelating agents (ethylene-diaminetetraacetic acid and ethylene glycol-bis(β-amino-ethylether) N,N,N′,N′-tetraacetic acid). The ECP obtained from each medium was not lethal to the groupers suggesting that the bacterium is low virulent. As grouper is carnivorous, therefore, the role of the protease played in the fish intestine probably is competing for nutrients and/or associated with the cause of edema leading to gastroenteritis.

Introduction

Vibriosis is one of the major infectious diseases in cultured marine fish and shellfish worldwide (Egidius, 1987; Lightner, 1988; Austin and Austin, 1993). However, only a few reports have been published on grouper vibriosis particularly in the Far East (Wong and Leong, 1990; Lee, 1995), and the causative agents were identified as *Vibrio alginolyticus*, *V. parahaemolyticus* and *V.* sp. Recently, outbreaks of mass mortality among the cultured groupers, manifesting swollen intestine with transparent yellow fluid (gastroenteritis), occurred in Taiwan (Yii *et al.,* 1997). *Vibrio carchariae* was isolated and identified as the causative agent of the syndrome(Yii *et al.,* 1997).

Extracellular virulence factors including proteases produced by *Vibrio* species are suggested to play a significant role in pathogenesis (Inamura *et al.,* 1985; Egidius, 1987; Nottage and Birkbeck, 1987a, b; Norqvist *et al.,* 1990; Farrell and Crosa, 1991; Stensvag *et al.,* 1993; Morita *et al.,* 1994; Lee, 1995; Lee *et al.,* 1997; Liu *et al.,* 1997). In the present study, we investigate the protease activities and virulence of the extracellular products of *V. carchariae* strain EmI82KL cultured on various media.

Materials and Methods

Bacterium, media and extracellular products (ECP)

Vibrio carchariae strain EmI82KL, originally isolated from the transparent yellow fluid of the

Abbreviations: ECP, extracellular products; 3,4-DCI, 3,4-dichloroisocoumarin; EDTA, ethylenediaminetetraacetic acid; EDDA, ethylenediamine di(o-hydroxyphenylacetic acid); EGTA, ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid; HPA, hide powder azure; PBS, phosphate buffered saline; PCMB, *p*-chloromercuribenzoate; PCMBs, *p*-chloromercuribenzene-sulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; TLCK, N-α-*p*-tosyl-l-lysine-chloromethylketone; TPCK, N-tosyl-l-phenyl-alanine chloromethylketone; TSA, tryptic soy agar.

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swollen intestine of diseased grouper (*Epinephelus coioides*) in Taiwan in 1993, was used in this study (Yii *et al.*, 1997). Stock cultures of strain EmI82KL were grown on tryptic soy agar (TSA; Oxoid, Basingstoke, supplemented with 2% NaCl) for 24 h at 30° C. Two swabs of these bacteria suspended in 5 ml phosphate buffered saline (PBS) pH 7.2, were spread onto each agar medium (+ 2% NaCl) overlaid with sterile cellophane and grown for 48 h at 30° C separately. The compositions of each agar medium were indicated in Table I. Each ECP was harvested as method previously described (Lee and Ellis, 1990). Briefly, 15 ml of PBS was added to the surface of the cellophane overlaying agar medium (+ 2% NaCl) and spread completely. The harvested bacterial suspension was then centrifuged at 25,000 *g* for 60 min at 4° C; the pellet was discarded. The supernatant fluids were passed through a 0.22-μm filter (Millipore, Bedford), and the ECP was stored in 1-ml aliquots at −70° C.

Protein and protease activity assays

Total protein was measured by the method of Bradford (1976) with bovine serum albumin as a standard. Protease activity was measured by hide powder azure (HPA) digestion as previously described (Lee and Ellis, 1990). Briefly, ECP solution (0.1 ml) was incubated and shaken with 25 mg HPA in 2.4 ml PBS at 37 °C for 15 min. On addition of 2.5 ml 10% trichloroacetic acid (TCA) and after centrifugation the absorbance of the supernatant was measured at 600 nm. Blanks were prepared by addition of 10% TCA to substrate prior to the proteolytic assay. One unit of protease activity is an increase in absorbance of 0.01 unit.

Fish and virulence tests

Groupers (*Epinephelus coioides* Hamilton, 1822), weighing about 30 g, were purchased from a farm in southern Taiwan. The fish were held in tanks (2,500 litre) supplied with air-lifted 3% salinity sea water at 25–28° C in our aquarium and were fed daily with commercial fish feed. The lethal tests, with batches of five fish per treatment (in duplicate), were conducted by intraperitoneal (i.p.) injection of the ECP (0.04 ml ECP/g fish body weight) into the fish. Sterile PBS was injected into the controls. Deaths of the tested animals were recorded daily for 1 week after i.p. injection.

Protease inhibition

The effects of 3,4-dichloroisocoumarin (3,4-DCI, Boehringer Mannheim GmbH, Mannheim), ethylenediaminetetraacetic acid (EDTA, Sigma), ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA, Sigma), iodoacetamide (Serva, Heidelberg), *p*-chloromercuribenzoate (PCMB, Sigma), *p*-chloromercuribenzene-sulfonic acid (PCMBs, Sigma), phenylmethanesulfonyl fluoride (PMSF, Sigma), *Nα-p*-tosyl-l-lysine-chloromethylketone (TLCK, Sigma), *N*-tosyl-l-phenylalanine chloromethylketone (TPCK, Sigma), sodium dodecyl sulfate (SDS, Serva), CuCl2 (Merck, Darmstadt), ZnCl2 (Merck) on the protease activity were examined. The PMSF and TPCK were dissolved in isopropanol (Riedel-de Haen S. A., Seelze), 3,4-DCI was dissolved in dimethylformamide (DMF; Serva) and the others were dissolved in distilled water. The concentration of each reagent was 10 mM as indicated in Table II. The controls contained only the solvents. After incubation at 37° C for 60 min, the changes of protease activity were determined using HPA assay as described above.

Results and Discussion

The ECP of *Vibrio carchariae* strain EmI82KL were harvested after 48 h of incubation of the culture at 30° C. The total protein and protease activity of each ECP was shown in Table I. The bacteria grown on peptone agar, nutrient agar or brain heart infusion agar manifested higher protease activity than that on tryptic soy agar (TSA) in terms of protein content. The addition of ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA), horse serum or mannose in the TSA enhanced the production of protease activity while the addition of grouper serum apparently reduced the enzyme activity. The use of minced grouper meat or peptone as a single nutrient source in agar remarkably enhanced the production of protease activity. No mortality was observed in all the treatment i.p. injected with each ECP or PBS (control).

The protease of the ECP was inhibited by two serine protease inhibitors, 3,4-DCI and PMSF (Table II). About 80% of the protease activity was

Table I. Protease activities of *Vibrio carchariae* strain EmI82KL grown on different culture media at 30 °C for 48 h.

Culture medium*	Total protein [mg/ml]	Protease activity** (HPA units/mg protein)
Tryptic soy agar (TSA)	1.34	291
TSA-EDDA	0.82	1179
TSA-HS	0.49	1022
TSA-GS	1.04	153
Nutrient agar	0.22	700
Brain heart infusion agar	0.50	642
PA	0.38	824
Agar-GM	0.42	1823

* TSA-EDDA: TSA containing ethylenediamine di-(o-hydroxyphenylacetic acid) (0.175 g/l).
TSA-HS: TSA containing 1% horse serum.
TSA-GS: TSA containing 1% grouper serum.
PA: Bacto-agar (1.5%) containing 1% Bacto-peptone.
Agar-GM: Bacto-agar (1.5%) containing 10% wet grouper meat.
All the agar media were supplemented with 2% NaCl and the tests were performed in duplicate.
** Hide powder azure (HPA) was used as the substrate for protease assay.

Table II. Effects of various reagents on the protease activity of ECP produced by *Vibrio carchariae* strain EmI82KL.

Reagent	Concentration [mM]	Relative activity (% of control)
None	0	100**
Cysteine protease inhibitor:		
Iodoacetamide	10	98
p-Chloromercuribenzoate	10	113
p-Chloromercuribenzene-sulfonic acid	10	90
Serine protease inhibitor:		
3,4-dichloroisocoumarin (3,4-DCI)	10	0
Phenylmethanesulfonyl fluoride	10	0
Chelating agent:		
EDTA*	10	20
EGTA	10	22
Broad-spectrum protease inhibitor:		
TLCK	10	61
TPCK	10	66
Other reagent:		
Sodium dodecyl sulfate	10	68
Divalent metal ion:		
$CuCl_2$	10	94
$ZnCl_2$	10	76

* EDTA: ethylenediaminetetraacetic acid.
EGTA: ethylene glycol-bis(β-amino-ethylether) N,N,N′,N′-tetraacetic acid.
TLCK: N-α-*p*-tosyl-l-lysine-chloromethylketone.
TPCK: N-tosyl-l-phenyl-alanine chloromethylketone.
** Hide powder azure was used as the substrate. The protease rate for the 100% control was 590 HPA digestibility units.

also inhibited by two chelating agents, EDTA and EGTA. The ECP protease was not or only partially inhibited by the other reagents used.

As the bacteria grown on peptone agar, nutrient agar or brain heart infusion agar produced higher protease activity than it grown on TSA in terms of protein content (Table I), TSA might not be a suitable medium for the bacterium to produce protease. In iron-limited condition (TSA+EDDA), the bacteria produced high protease activity compared to normal condition (TSA). Presumably the increase of protease production might be advantageous for the bacteria to acquire essential iron from iron-binding proteins as that reported by Hirst and Ellis (1996). The addition of horse serum manifested higher ECP protease activity than the addition of grouper serum in the TSA indicating the presence of protease inhibitor(s) in the fish serum. In addition, the use of grouper meat or peptone as a single nutrient source remarkably enhanced ECP protease activity. It seems that the addition of proteinaceous materials from animal sources (i.e. horse serum, grouper meat or peptone) is more appropriate than that from plant source (TSA) for the bacteria to produce higher amount of protease. Although the role of the protease playing in the disease process is still not clear, the above results may be helpful in explaining the existence, survival and infection of the bacteria in the intestine of the carnivorous groupers.

Vibrio carchariae has been first isolated from a dead sandbar shark (*Carcharhinus plumbeus*) at the National Aquarium in Baltimore, U. S. A., in 1982 and decribed to be associated with mortality of captive sharks (Grimes *et al.*, 1984). In a further report, Grimes and colleagues (1985) failed to reproduce clinically observable disease when infecting healthy lemon sharks (*Negaprion brevirostris*) with *V. carchariae*. Recently, the same *Vibrio* species was isolated and identified as the causative agent of gastroenteritis with a swollen intestine containing transparent yellow fluid in the groupers *Epinephelus coioides* although a high LD_{50} value (low virulence) was obtained (Yii *et al.*, 1997). In the present study, the ECP of the bacterium grown on various media (with high or low protease activity, see Table I) was also found to be low virulent after i.p. injection indicating that the ECP protease may not be a toxic factor although some *Vibrio* proteases have been recognized as toxins (Ina-

mura *et al.*, 1985; Nottage and Birkbeck, 1987a, b; Lee, 1995; Lee *et al.*, 1997).

The protease of the ECP was characterized as a metal-chelator-sensitive serine protease for its inhibition by serine protease inhibitors (3,4-DCI and PMSF) and chelating agents (EDTA and EGTA). To our knowledge, there has no report concerning serine protease of *V. carchariae* been published.

Since protease has been regarded as one of the potential invasive factors produced by *V. carchariae* (Grimes *et al.*, 1989), the role of it playing in the intestine of carnivorous grouper may be competing for nutrients (such as nitrogens) and/or associated with the resultant edema as that well demonstrated by Maeda and Yamamoto (1996) in a review concerning pathogenic mechanisms induced by microbial proteases in microbial infections. The role of this serine protease playing in the pathogenesis of gastroenteritis in the grouper is now under investigation.

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