Lethal Attribute of Serine Protease Secreted by *Vibrio alginolyticus* Strains in Kuruma Prawn *Penaeus japonicus*

Ferng-Ruey Chen, Ping-Chung Liu and Kuo-Kau Lee*

Department of Aquaculture, National Taiwan Ocean University, 2, Pei-Ning Road, Keelung, Taiwan. Fax: 008862 24633150. E-mail: kklee@ntou66.ntou.edu.tw

* Author for correspondence and reprint requests

Z. Naturforsch. **55c,** 94–99 (2000); received September 9/November 8, 1999

Extracellular Products, Lethality, *Penaeus japonicus*, Serine Protease, *Vibrio alginolyticus*

Toxicity of the extracellular products (ECP) and the lethal attribute of serine protease secreted by five pathogenic *Vibrio alginolyticus* strains from various sources in kuruma prawn *Penaeus japonicus* were studied. The ECPs of organisms originally isolated from diseased kuruma prawn or small abalone *Haliotis diversicolor supertexta* were more lethal (LD_{50} value of 0.48 or 0.41 μg protein/g prawn) than those from diseased tiger prawn *P. monodon*, yellowfin porgy *Acanthopagrus latus* or horse mackerel (LD_{50} value of 0.98–1.17 μg protein/g prawn). All the ECPs manifested strong, weak and no activities against gelatin, sheep erythrocytes and chitin, respectively. In immunodiffusion tests using rabbit antiserum to a purified 33 kDa serine protease of strain Swy against ECP of each tested strain produced one single precipitation band in each treatment. Furthermore, the serine protease was suggested to be the dominant protease secreted by *V. alginolyticus* strains tested since the majority of enzymatic activity of the respective ECP was inhibited by phenylmethanesulfonyl fluoride (PMSF). A higher inhibition of serine protease activity by PMSF resulted in lower mortality rate of the ECPs injected into the prawns suggesting that the protease is one of the major lethal factor(s) secreted by *V. alginolyticus.*

Introduction

Vibriosis is one of the major infectious diseases associated with mass losses in marine cultured penaeids worldwide (Lightner, 1988; Vera *et al.*, 1992; Hameed and Rao, 1994; Pizzutto and Hirst, 1995; Lee *et al.*, 1996a, b; Liu *et al.*, 1996a, b; Alvarez *et al.*, 1998; Karunasagar *et al.*, 1998). Recently, *Vibrio alginolyticus* has been frequently isolated from diseased prawns and is virulent to the two major cultured penaeids, *Penaeus monodon* and *P. japonicus*, in Taiwan (Lee *et al.*, 1996a, b).

Extracellular virulence factors including proteases produced by various *Vibrio* species isolated from diseased fish and shellfish are suggested to play a significant role in pathogenesis (Inamura *et al.*, 1985; 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). A 33 kDa serine protease produced by *V. alginolyticus* strain Swy has recently been purified and characterised as a lethal toxin in *P. japonicus* (Lee *et al.*, 1997; Chen *et al.*, 1999).

In the present paper, the toxicity of the extracellular products (ECP) and role of extracellular serine protease produced by five strains of *V. alginolyticus*, isolated from marine fish and shellfish, in *P. japonicus* are investigated and discussed.

Abbreviations: ECP, extracellular products; HPA, hide powder azure; PBS, phosphate buffered saline; PMSF, phenylmethanesulfonyl fluoride; RαP, rabbit antiserum to a purified serine protease; TCA, trichloroacetic acid.

Materials and Methods

Bacteria

One reference strain and four field isolates of *Vibrio alginolyticus* were used in this study. The reference strain was ATCC 17749 (Sakazaki, 1968) and was originally isolated from horse mackerel. The field isolates were obtained from diseased kuruma prawn *P. japonicus* (strain Swy), tiger prawn *P. monodon* (strain Val), yellowfin porgy *Acanthopagrus latus* (strain RGSW) and small abalone *Haliotis diversicolor supertexta* (strain H-11) in Taiwan. The source of specimen, origin and date of isolation are provided in Table I. The bacterial cultures were stored in phosphate-buf-

0939–5075/2000/0100–0094 $ 06.00

Table I. *Vibrio alginolyticus* strains used in this study.

Strain	Origin	Isolated organ	Locality	Year/isolation	Reference
ATCC17749	Horse mackerel	UN	UN	UN	Sakazaki, 1968
Swy	*Penaeus japonicus*	hepatopancreas	I-Lan	1993	Lee *et al.*, 1996a
Val	*P. monodon*	haemolymph	I-Lan	1994	Lee *et al.*, 1996b
H-11	*Haliotis diversicolor supertexta*	haemolymph	Kaohsiung	1998	this study
RGSW	*Acanthopagrus latus*	ascites	Tainan	1997	this study

ATCC: American Type Culture Collection, Rockville, Maryland, USA.
UN: unknown.

fered saline (PBS, pH 7.2) containing 10% glycerol at −70 °C.

Extracellular products (ECP)

Stock cultures of each strain were grown on tryptone agar (Difco, Detroit MI) (TA; contained 1.8% tryptone, 1.5% agar and 2.5% NaCl) plates for 24 h at 30 °C and two swabs of each bacteria were suspended in 10 ml of PBS. The suspension was spread onto TA overlaid with sterile cellophane and incubated for 24 h at 30 °C. Each ECP was harvested following a method described previously (Lee and Ellis, 1990). In brief, 5 ml of PBS was added to the surface of the cellophane overlaying TA 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 −20 °C. Total protein was measured following the method of Bradford (1976) with bovine serum albumin as a standard.

Enzymatic and haemolytic activities of ECP

Protease activity was measured by hide powder azure (HPA) digestion as described previously (Lee and Ellis, 1990). Briefly, 0.1 ml enzyme solution was incubated with 25 mg HPA in 2.4 ml PBS at 37 °C for 15 min with shaking. On addition of 2.5 ml 10% trichloracetic acid (TCA) and after centrifugation the absorbance of the supernatant was measured at 600 nm. Controls were prepared by the addition of TCA to HPA in the absence of the enzyme. One unit of protease activity is an increase in absorbance of 0.01 unit. Caseinase, gelatinase, phospholipase, lipase and chitinase activities of each ECP were observed by placing 20 μl samples of ECP in wells cut in agarose (1% in PBS) that contained either 1% sodium caseinate, 0.5% gelatin, 0.2% egg yolk, 1% Tween 80 or 0.3% chitin, respectively. The plates were incubated in a humidified chamber for 24 h at 27 °C. The diameter of the lytic halo around each well was measured. Haemolytic activity was measured by a standard microtitration method with sheep erythrocytes as described previously (Hastings and Ellis, 1985). All the tests were performed in duplicate.

Rabbit antiserum to the purified serine protease of strain Swy

The purification of a 33 kDa serine protease from ECP of *V. alginolyticus* strain Swy, using ÄKTA *purifier* System with hydrophobic interaction chromatography, anion exchange and gel filtration columns, is described and the protease characterised as a lethal toxin elsewhere (Chen *et al.*, 1999). The purified protease (120 μg protein/ml) was formalinised with 3% formalin for 48 h prior to the immunisation in a New Zealand white rabbit weighing about 2 kg. The formalinised protease was further dialysed against PBS for 48 h at 4 °C. Two millilitres of the dialysed preparation were emulsified in an equal volume of Freund's complete adjuvant and injected into the rabbit subcutaneously. Six weeks later, the rabbit was boosted with the same antigen emulsified in Freund's incomplete adjuvant as that described above. Rabbit antiserum to the purified serine protease (RαP) was obtained 2 weeks later. Aliquots of 1 ml of the antiserum were stored at −70 °C.

Immunodiffusion

Ouchterlony double-diffusion gels were prepared by layering a 1.5% agarose (in PBS) on Petri dish. For examining immuno- or cross-reactivity of RαP with ECPs from all five strains, the centre well was filled with RαP and the ECPs were placed in the surrounding 5 wells. Twenty μl of RαP or ECPs were placed in each well and the gels were allowed to react in a humidified chamber at 25 °C for 24 h, and then scored for the occurrence of immunoprecipitation band.

Inhibition of protease activity by PMSF

The effect of 5 mM phenylmethanesulfonyl fluoride (PMSF; Sigma, dissolved in isopropanol) on the protease activity was examined. After incubation at 37 °C for 60 min, the changes of protease activity were determined using the HPA assay as described above.

Toxicity in prawn and its inhibition

Kuruma prawn (*Penaeus japonicus*), weighing about 10 g, were purchased from a farm in southern Taiwan. The prawns 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 prawn feed. The lethal tests, with batches of ten prawns per treatment in duplicate, were conducted by intramuscular (i.m.) injection of 0.1 ml of samples into the prawn at the site between 4th and 5th abdominal segments (Trevors and Lusty, 1985; Vera *et al.*, 1992). Sterile PBS and 20 mM PMSF (3:1, v/v) were accordingly injected i.m. into parallel controls. Protease inhibitor (20 mM PMSF) was added in each ECP (3-fold LD_{50} value) (1:3, v/v) and incubated at 37 °C for 1 hour prior to the investigation of the inhibition of toxicity in *P. japonicus*. The protocol of toxicity inhibition tests was the same as described above in the LD_{50} tests. The mortalities of all the tested animals were recorded for 2 days post injection.

Results

The ECP of *Vibrio alginolyticus* strains ATCC 17749, Swy, Val, H-11 or RGSW was harvested after 24 h of incubation of the culture at 30 °C. The total protein, enzymatic and haemolytic activities of each ECP are shown in Table II. The ECP of strains Swy, H-11 or RGSW manifested stronger proteolytic (5630–6983 HPA units/mg protein) and caseinase activities than those of the other two strains. Low or no activities of chitinase, phopholipase, lipase or haemolysin were detected in the ECP of each different strain.

Only one single immunoprecipitation band (arc) in Ouchterlony double-diffusion gels was observed using RαP against ECP of strain Swy as well as against ECP from other four strains (data not shown).

ECPs were found to be lethal to *P. japonicus* with LD_{50} values ranging from 0.41 to 1.17 μg protein/g prawn body weight. The ECP of strains Swy and H-11 were more lethal than those of other three strains tested in terms of LD_{50} values (Table II).

The protease activity in each ECP (3-fold of each LD_{50} value) was completely inhibited by 5 mM PMSF (a serine protease inhibitor) (Ta-

Table II. Characteristics and pathogenicity of extracellular products in *Penaeus japonicus* for selected *Vibrio alginolyticus* isolates.

Strain	Protein (mg/ml)	Protease activity (HPA units/ mg protein)	Protease against		Phospholipase against egg yolk	Lipase against Tween 80	Chitinase against chitin	Haemolysis against erythrocytes from sheep	LD_{50} (μg/g prawn)
			Casein	Gelatin					
ATCC17749	0.28	3579	++	++++	–	+	–	7.1	0.98
Swy	0.12	6983	++++	++++	++	–	–	33.3	0.48
Val	0.21	4224	++	++++	–	+	–	9.8	1.17
H-11	0.14	5630	+++	++++	–	+	–	29.6	0.41
RGSW	0.18	6120	++++	++++	++	–	–	11.4	1.07

Ratio of hydrolysis halo to cut well diameter (mm): ++++, 16–20; +++, 11–15; ++, 6–10; +, 1–5; –, 0.
HPA, hide powder azure.
All the tests were conducted in duplicate.

ble III), indicating that the major protease in the ECP produced by each *V. alginolyticus* strain tested is a serine protease.

Three folds of the LD_{50} value of each ECP were used in the lethal toxicity inhibition tests, and the toxicity of each preparation in the *P. japonicus* was partially or completely inhibited by the presence of 5 mM PMSF (Table III). All the prawns were killed after injection with 3-fold LD_{50} value of each ECP in the absence of PMSF. No mortality was observed in all the controls injected with PBS and PMSF.

Discussion

Pathogenic *Vibrio alginolyticus* has been previously described in the gilthead sea bream *Sparus aurata* (Colorni *et al.*, 1981), sea mullet *Mugil cephalus* (Burke and Rodgers, 1981), turbot *Scophthalmus maximus* (Austin *et al.*, 1993), grouper *Epinephelus malabaricus* (Lee, 1995), bivalve molluscs (Nottage and Birkbeck, 1986) and the rotifer *Brachionus plicatilis* (Yu *et al.*, 1990). Recently, the pathogen has also been recorded in penaeids (Lee *et al.*, 1996a, b; Karunasagar *et al.*, 1998), the Catarina scallop *Argopecten ventricosus* (=*circularis*) (Sainz *et al.*, 1998) and the red abalone *Haliotis rufescens* (Anguiano-Beltran *et al.*, 1998). Thus prompted us to investigate the lethal toxicity of the ECP produced by four different pathogenic strains recently isolated from fish and shellfish by our laboratory in Taiwan in *P. japonicus*.

No chitinase activity was detected in each ECP which may be due to the absence of chitin in the medium used to grow each strain. In the present study, the ECPs (of strains Swy and H-11) with higher protease activities were more lethal than those with lower protease activities (of strains ATCC 17749 and Val) except that of strain RGSW. The reason for a higher protease activity with a lower lethal toxicity in the ECP of strain RGSW is not clear. Presumably, this may be due to the host being less susceptible to the protease of strain RGSW or derived from other unknown components in the ECP which may affect the lethal toxicity of the protease in the prawn.

Table III. Neutralisation of ECP lethal toxicity for *Penaeus japonicus* by incubation with PMSF (final concentration of PMSF in each related treatment was 5 mM); 0.1 ml of sample was intramuscularly injected into the animal weighing about 10 g.

Treatment	Dose (μg protein/g prawn)	Mortality (%)	Relative protease activity (%)
PBS + PMSF (3:1, v/v)	0	0	–
ATCC17749 ECP + PBS (3:1, v/v)	2.94	100	100
ATCC17749 ECP + PMSF (3:1, v/v)	2.94	50	10
Swy ECP + PBS (3:1, v/v)	1.44	100	100
Swy ECP + PMSF (3:1, v/v)	1.44	10	4
Val ECP + PBS (3:1, v/v)	3.51	100	100
Val ECP + PMSF (3:1, v/v)	3.51	60	17
H-11 ECP + PBS (3:1, v/v)	1.23	100	100
H-11 ECP + PMSF (3:1, v/v)	1.23	0	3
RGSW ECP + PBS (3:1, v/v)	3.21	100	100
RGSW ECP + PMSF (3:1, v/v)	3.21	30	5

The treatments were incubated at 37 °C for 1 hour prior to injection.
All the tests were conducted in duplicate and recorded for 2 days.
Relative protease activity (%) represented the residual activity of each ECP pre-treated with PMSF over the protease activity of respective ECP added with PBS, and the respective activity of the latter was used as control (100% of relative protease activity).

In a previous investigation, both the live bacteria and ECP of strain Val were found to be more virulent or lethal in tiger prawn *P. monodon* than in kuruma prawn *P. japonicus* (Lee *et al.*, 1996b). In addition, live bacteria of strain Swy were more virulent in *P. japonicus* than in *P. monodon* (Lee *et al.*, 1996a). Therefore, ECP of isolate RGSW obtained from *A. latus* (Table I) manifesting lower lethal toxicity in *P. japonicus* (Table II) may be not uncommon.

Inhibition of protease activity by PMSF, as shown *in vitro*, resulted in a lower mortality *in vivo* (Table III). Extracellular serine protease of strain Swy was previously characterised as a lethal toxin in *P. japonicus* (Lee *et al.*, 1997; Chen *et al.*, 1999). In the present investigation the inhibition of extracellular serine protease activity from other sources was also found affecting the lethal toxicity of respective ECP in the prawns. Serine protease is confirmed to be a major toxin in the ECP of strains Swy and H-11 since almost all the lethal toxicity of both ECPs was inhibited by the presence of PMSF. However, other unknown toxic component(s) in addition to serine protease may also be present in the ECPs of strains ATCC 17749, RGSW or Val since lethal toxicity of the respective ECPs was not completely inhibited by the presence of PMSF even with a high-inhibition (more than 83%) of protease activity (see Table III).

Since the similarities in LD_{50} and lethal toxicity inhibition tests between ECPs of strains Swy and H-11 found here (Tables II and III), the transmission of virulent strains between hosts, i.e. *P. japonicus* and *Haliotis diversicolor supertexta*, could occur as strain H-11 was originally isolated from diseased *H. diversicolor supertexta* in Kaohsiung (Table I), where most of the *P. japonicus* larvae in Taiwan are propagated. A further study on the characteristics and pathogenesis of these two isolates is now under way.

Proteases produced by bacteria have been known as important virulence factors in many diseases (Maeda and Yamamoto, 1996). Quite a few proteases produced by *V. alginolyticus* have been characterised as toxins in fish and shellfish (Nottage and Birkbeck, 1987a, b; Lee, 1995; Lee *et al.*, 1997; Chen *et al.*, 1999). In the present work, we were able to determine that the ECPs from strains of *V. alginolyticus* recently isolated from fish and shellfish shared some similarities both in terms of the antigenic relationship of serine protease and the inhibition of serine protease activity. Although an important attribute of serine protease to the lethal toxicity of ECP produced by *V. alginolyticus* from various sources in *P. japonicus* was confirmed in the present study, definite conclusions as to the toxic nature of the protease may require assay of more highly purified protein.

Acknowledgements

This study was supported by the grant NSC 88–2313-B-019–034, from the National Science Council, R. O. C. We thank Mr. Tu C. C. for his technical assistance, and anonymous referees for their helpful comments and suggestions during the course of this work.

Alvarez J. D., Austin B., Alvarez A. M. and Reyes H. (1998), *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. J. Fish Dis. **21,** 313–316.

Anguiano-Beltran C., Searcy-Bernal R. and Lizarraga-Partida M. L. (1998), Pathogenic effects of *Vibrio alginolyticus* on larvae and postlarvae of the red abalone *Haliotis rufescens*. Dis. Aquat. Org. **33,** 119–122.

Austin B., Stobi M., Robertson P. A. W., Glass H. G., Stark J. R. and Mudarris M. (1993), *Vibrio alginolyticus*: the cause of gill disease leading to progressive low-level mortalities among juvenile turbot, *Scophthalmus maximus* L., in a Scottish aquarium. J. Fish Dis. **16,** 277–280.

Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Burke J. and Rodgers L. (1981), Identification of pathogenic bacteria associated with the occurrence of 'red spot' in sea mullet, *Mugil cephalus* L., in south-eastern Queensland. J. Fish Dis. **3,** 153–159.

Chen F. R., Liu P. C. and Lee K. K. (1999), Purification and partial characterization of a toxic serine protease produced by pathogenic *Vibrio alginolyticus*. Microbios **98,** 95–111.

Colorni A., Paperna I. and Gorgin H. (1981), Bacterial infections in gilthead sea bream *Sparus aurata* cultured at Elat. Aquaculture **23,** 257–260.

Farrell D. H. and Crosa J. H. (1991), Purification and characterization of a secreted protease from the pathogenic marine bacterium *Vibrio anguillarum*. Biochemistry **30,** 3432–3436.

Hameed A. S. S. and Rao P. V. (1994), Studies on the chemical control of a *Vibrio campbellii*-like bacterium affecting hatchery-reared *Penaeus indicus*. Aquaculture **127,** 1–9.

Hastings T. S. and Ellis A. E. (1985), Differences in the production of haemolytic and proteolytic activities by various isolates of *Aeromonas salmonicida*. In: Fish and Shellfish Pathology. (A. E. Ellis, ed.). Academic Press, London, pp 69–77.

Inamura H., Nakai T. and Muroga K. (1985), An extracellular protease produced by *Vibrio anguillarum*. Bull. Japan. Soc. Sci. Fish. **51,** 1915–1920.

Karunasagar I., Otta S. K. and Karunasagar I. (1998), Disease problems affecting cultured penaeid shrimp in India. Fish Pathol. **33,** 413–419.

Lee K. K. (1995), Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus,* Bloch et Schneider. Microb. Pathog. **19,** 39–48.

Lee K. K. and Ellis A. E. (1990), Glycerophospholipid: cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytolysin of *Aeromonas salmonicida*. LPS stabilizes and enhances toxicity of the enzyme. J. Bacteriol. **172,** 5382–5393.

Lee K. K., Yu S. R., Yang T. I., Liu P. C. and Chen F. R. (1996a), Isolation and characterization of *Vibrio alginolyticus* isolated from diseased kuruma prawn, *Penaeus japonicus*. Lett. Appl. Microbiol. **22,** 111–114.

Lee K. K., Yu S. R., Chen F. R., Yang T. I. and Liu P. C. (1996b), Virulence of *Vibrio alginolyticus* isolated from diseased tiger prawn, *Penaeus monodon*. Curr. Microbiol. **32,** 229–231.

Lee K. K., Yu S. R. and Liu P. C. (1997), Alkaline serine protease is an exotoxin of *Vibrio alginolyticus* in kuruma prawn, *Penaeus japonicus*. Curr. Microbiol. **34,** 110–117.

Lightner D. V. (1988), Vibrio diseases of penaeid shrimps. In: Disease Diagnosis and Control in North American Marine Aquaculture and Fisheries Science. Vol 6, (C. J. Sinderman and D. V. Lightner, eds.). Elsevier, Amsterdam, pp 42–47.

Liu P. C., Lee K. K. and Chen S. N. (1996a), Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*. Lett. Appl. Microbiol. **22,** 413–416.

Liu P. C, Lee K. K, Yii K. C., Kou G. H and Chen S. N. (1996b), Isolation of *Vibrio harveyi* from diseased kuruma prawns *Penaeus japonicus*. Curr. Microbiol. **33,** 129–132.

Liu P. C., Lee K. K., Tu C. C. and Chen S. N. (1997), Purification and characterization of a cysteine protease produced by pathogenic luminous *Vibrio harveyi*. Curr. Microbiol. **35,** 32–39.

Maeda H. and Yamamoto T. (1996), Pathogenic mechanisms induced by microbial proteases in microbial infections. Biol. Chem. Hoppe-Seyler **377,** 217–226.

Morita J., Suzuki S. and Kusuda R. (1994), Protease production profiles of the fish pathogen *Listonella anguillara* based on substrate specificities. Fish. Sci. **60,** 741–745.

Norqvist A., Norrman B. and Wolf-Watz H. (1990), Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum*. Infect. Immun. **58,** 3731–3736.

Nottage A. S. and Birkbeck T. H. (1986), Toxicity to marine bivalves of culture supernatant fluids of the bivalve-pathogenic *Vibrio* strain NCMB 1338 and other marine vibrios. J. Fish Dis. **9,** 249–256.

Nottage A. S. and Birkbeck T. H. (1987a), Purification of a proteinase produced by the bivalve pathogen *Vibrio alginolyticus* NCMB 1339. J. Fish Dis. **10,** 211–220.

Nottage A. S. and Birkbeck T. H. (1987b), Production of proteinase during experimental infection of *Ostrea edulis* L. larvae with *Vibrio alginolyticus* NCMB 1339 and the antigenic relationship between proteinase produced by marine vibrios pathogenic for fish and shellfish. J. Fish Dis. **10,** 265–273.

Pizzutto M. and Hirst R. G. (1995), Classification of isolates of *Vibrio harveyi* virulent to *Penaeus monodon* larvae by protein profile analysis and M13 DNA fingerprinting. Dis. Aquat. Org. **21,** 61–68.

Sainz J. C., Maeda-Martinez A. N. and Ascencio F. (1998), Experimental vibriosis induction with *Vibrio alginolyticus* of larvae of the Catarina scallop *Argopecten ventricosus* (=*circularis*) (Sowerby II, 1842). Microb. Ecol. **35,** 188–192.

Sakazaki R. (1968), Proposal of *Vibrio alginolyticus* for biotype 2 *Vibrio parahemolyticus*. Japan. J. Med. Sci. Biol. **21,** 359–362.

Stensvag K., Jorgensen T. O., Hoffman J., Hjelmeland K. and Bogwald J. (1993), Partial purification and characterization of extracellular metalloproteases with caseinolytic, aminopeptidolytic and collagenolytic activities from *Vibrio anguillarum*. J. Fish Dis. **16,** 525–539.

Trevors J. V. and Lusty C. W. (1985), A basic microcomputer program for calculating LD_{50} values. Water Air Soil Pollut. **24,** 431–442.

Vera P., Navas J. I. and Quintero M. C. (1992), Experimental study of the virulence of three species of *Vibrio* bacteria in *Penaeus japonicus* (Bate 1881) juveniles. Aquaculture **107,** 119–123.

Yu J. P., Hino A., Noguchi T. and Wakabayashi H. (1990), Toxicity of *Vibrio alginolyticus* on the survival of the rotifer *Brachionus plicatilis*. Nippon Suisan Gakkaishi **56,** 1455–1460.