Short Paper

The Protease-producing Ability of Vibrios Isolated from Larvae and Juveniles of Japanese Flounder

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Abstract: A total of 780 vibrios were isolated from Japanese flounder, Paralichthys olivaceus, at different stages of development and examined for the protease-producing ability of each isolate. The highest and lowest values in mean enzyme activities of isolates were observed on days 109 and 67 after hatching, respectively. High abilities (>1.00 U/µg) were detected in the isolates from fish on days 3, 23, and 45 after hatching, while as many as 62% of all vibrios showed low potential of production (<0.10 U/µg). Inhibitory effects of PMSF and OPA were found in 61 and 50% of all Vibrio isolates, respectively. These results showed that larvae and juveniles of Japanese flounder harbor the vibrios capable of producing various types of proteases at different activities, in their intestines.

Key words: Vibrio spp.; Protease; Japanese flounder; Virulence

It is well known that intestinal tracts of marine fish are colonized mainly by genus Vibrio regardless of species and developmental stages of host fish1). This fact suggests that there is a close relationship between marine fish and vibrios. Of members of genus Vibrio, some species, such as V. anguillarum, V. alginolyticus and V. vulnificus, are known as causative agents of vibriosis in aquatic animals including fish, crustaceans and mollusks, and they often cause serious losses in aquaculture industries. Among the extracellular products of pathogenic vibrios, protease has been considered as a virulence factor of vibrios2,3). However, little is known about the extracellular protease-producing ability of gut Vibrio from marine fish. In this study, therefore, the protease-producing ability of vibrios isolated from Japanese flounder, Paralichthys olivaceus, at different stages of development was examined for the better understanding of their virulence potential.

A total of 780 vibrios were isolated from the intestinal tract of larvae and juveniles of Japanese flounder on days 3, 23, 45, 67 and 109 after hatching by being incubated aerobically at 25°C for 7-8 days on 1/20PYBG, PYBG and TCBS agar media, and identified at the genus level as previously reported1). Each isolate was incubated in PYBG broth at 25°C for 48 h. After incubation, the culture was centrifuged twice at 9,600×g for 20 min (0°C) to obtain the culture supernatant fluid.

Protease activity in the culture supernatant fluid was measured by the casein degradation method of Gudmundsdóttir4), with some modifications. Two hundred and fifty µl of enzyme sample were incubated with 2,250 µl of 1% (w/v) azocasein (Sigma) in 60 mM phosphate buffer (pH 7.2) at 35°C for 60 min. In reagent blanks, 60 mM phosphate buffer was used instead of enzyme sample. The reaction was stopped by adding 2.5 ml of 10% (w/v) trichloroacetic acid. After being kept for 30 min at room temperature, the precipitate was removed by centrifugation, and a 2.5 ml aliquot of the supernatant fluid was added to an equal volume of 1.0 M NaOH. Released azodye was measured spectrophotometrically at 450 nm (A450) against a reagent blank. The assay was done in duplicate. One unit (U) of protease activity was defined as an increase of 0.001 in A450 under the assay conditions. The protein content was determined by a protein assay kit (Bio-Rad Laboratories), using bovine serum albumin (Sigma) as a standard. Solutions of 25 mM phenyl methyl sulphonyl fluoride (PMSF) and 50 mM 1,10-phenanthroline (OPA) dissolved in ethanol were used as inhibitors. Equal volumes of enzyme and inhibitor solutions were mixed and incubated at 35°C for 10 min prior to protease assays. Controls contained the solvent alone. The inhibition was deemed positive if >10% of the activity was reduced by the treatment4).

All Vibrio isolates produced the extracellular proteases with activities ranging from 0.01 to 18.71 U/µg, and the enzyme activity remarkably varied with bacterial isolates and developmental stages of Japanese flounder (Table 1). The highest (0.832 U/µg) and lowest (0.054 U/µg) values in mean enzyme activities of isolates were observed on day 109 and 67 days after hatching. High activities (>1.00 U/µg) were found in 1.5, 4.3 and 10.3% of the vibrios isolated from fish on days 23, 3 and 109 after hatching, respectively, but could not be detected on days 45 and 67.

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In addition, as many as 62.4% of all vibrios showed low potential of production (<0.10 U/μg).

On the other hand, inhibitory effects of PMSF and OPA were observed in 61 and 50% of all Vibrio isolates, respectively (Fig. 1). Thirty to 44% of vibrios isolated on each sampling day produced both PMSF- and OPA-sensitive protease (type A), and 7-29% of them produced PMSF-sensitive but OPA-insensitive protease (type B). Nine to 37% of vibrios produced OPA-sensitive but PMSF-insensitive protease (type C) while 8-30% of them produced both PMSF- and OPA-insensitive protease (type D). Composition profiles of isolates on days 3 and 23 may be different from those of other specimens. PMSF and OPA are known as serine protease and metalloprotease inhibitors, respectively. If all proteases from Vibrio isolates fall under these categories, 30-44% of vibrios (type A) produce both serine protease and metalloprotease, and possibly other proteases, too. These results indicate that each Vibrio isolate from Japanese flounder produced different types of proteases. Nottage and Birkbeck, and Norqvist et al. reported that metalloproteases were produced by V. alginolyticus and V. anguillarum. The present study shows that at least 30-44% of Vibrio isolates produce more than two different proteases, including serine protease and metalloprotease.

Table 2 shows that the mean enzyme activity varied with the media used for isolation of vibrios. Especially, vibrios with high abilities were isolated from only PYBG medium. This result reveals that the medium used for isolation remarkably influence not only the recovery efficiency of microflora but also the enzyme-producing ability of each isolate even within the same genus.

The present results show that larvae and juveniles of Japanese flounder harbor different vibrios capable of producing various types of protease in their intestines. As many as 62% of vibrios seem to be low potential of production. Contrary to this, 3% of Vibrio isolates could produce protease with high activities, which may work as a virulence factor. However, taxonomic status of these isolates remains to be studied. Further studies along this line should be undertaken in the near future.

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References