Characterization of Proteolytic and Collagenolytic Psychrotrophic Bacteria of Ice-Stored Freshwater Prawn, *Macrobrachium rosenbergii*

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ABSTRACT—A microbiological analysis of ice-stored freshwater prawn, Macrobrachium rosenbergii, *was conducted to enumerate, isolate, identify, and test the proteolytic and collagenolytic activities of the psychrotrophic, proteolytic microflora. Twenty proteolytic cultures were isolated from 4-day ice-stored prawns using Azocoll-supplemented (AZOPCA) medium. These isolates were identified as belonging to* Pseudomonas *group I (fluorescent) species,* Pseudomonas *group II (nonfluorescent) species,* Alteromonas (Pseudomonas) putrefaciens, Flavobacterium *species, and* Cytophaga *species. Four of the identified isolates showing rapid and extensive proteolysis on AZOPCA medium at 5°C and 30°C showed collagenolytic activities on insoluble bovine collagen and prawn tissues. Proteolytic and collagenolytic activities were higher at 8°C than at 1°C and 5°C. Collagenolytic activity was higher with prawn tissues as substrate than with insoluable bovine collagen. These results also indicate that the proteolytic and collagenolytic microflora contribute very slightly to the development of mushiness in the initial stage of storage on ice (2-3 days) but contribute more extensively after 4 days.*

Introduction

Freshwater prawn, *Macrobrachium rosenbergii* culture is a relatively new and rapidly expanding industry in Hawaii. Annual production increased from 4,900 kg in 1974 to 13,600 kg in 1980 (Shang, 1981) and to 150,000 kg in 1985 (Fassler[1]). With this production volume, effective and economic postharvest handling (processing) of the prawns becomes critical.

Quality deterioration of the raw prawn under iced or refrigerated storage is characterized by softening (mushiness) which causes loosening and flaking of the cooked tissue of the prawn tail when touched or rubbed (Nip et al., 1985a). This mushy texture develops within 2-3 days in iced storage and is most pronounced in the proximal (first) tail section (adjacent to the cephalothorax). It progresses (downward) to the other sections of the tail with prolonged iced storage.

Little research has been done on the development of mushiness in freshwater prawns or other shellfishes during iced storage. Results indicate that one possible cause is the activity of a collagenolytic enzyme released by the hepatopancreas during postmortem autolytic processes (Baranowski et al., 1984; Nip et al., 1985b). The other possible cause which has not yet been investigated is the enzymatic activity contributed by the psychrotrophic proteolytic and/or collagenolytic microflora of the ice-stored prawn.

Neither the role of proteolytic/collagenolytic psychrotrophic microflora in the development of mushiness in *M. rosenbergii* nor the microflora of freshwater prawn produced in Hawaii have been reported. No previous work other than total and/or proteolytic bacterial counts have appeared in the literature (Angel et al., 1981; Shepherd, 1979; Waters and Hale, 1981). Several investigators have suggested or found that predominantly gram-negative psychrotrophic microflora develop in ice-stored fish and shellfish (Hobbs, 1983; Hobbs and Hodgkiss, 1982; Lee and Pfeifer, 1975; Nickelson and Vanderzant, 1976; Van Spreekens, 1977). These organisms belong to the genera *Pseudomonas*, *Alteromonas*, *Moraxella*, and *Acinetobacter.* The first two are known to be active spoilage organisms having proteolytic ability (Hobbs and Hodgkiss, 1982; Kazanas, 1967). However, only certain strains of pigmented *Pseudomonas* have been reported to exhibit collagenolytic activity (Adamcic and Clark, 1970; Waldvogel and Swartz, 1969). The objective of this study was to isolate, enumerate, and identify proteolytic and collagenolytic, psychrotrophic bacteria of 4-day ice-stored prawns and to test the collagenolytic and proteolytic activities of the isolated bacteria quantitatively.

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[1]Fassler, R. Aquaculture Development Program, Hawaii Dep. Land Natural Resour. Personal commun., Jan. 1986.

Materials and Methods

Media

Total Plate Counts: AZOPCA (Isolation Medium)

Plate count agar supplemented with 0.25 percent Azocoll[2] (Calbiochem Co., San Diego, Calif.) was used to isolate and enumerate proteolytic bacteria of fresh and ice-stored freshwater

[2]Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

prawns. Azocoll powder was sterilized by exposure of a 50 mg sample to 25 ml propylene oxide (Baranowski et al., 1984). Sterile Azocoll powder was mixed into sterile plate count agar (cooled to 50°C) before pouring into plates.

PCAP Medium

Plate count agar supplemented with 0.5 percent peptone (Difco, Detroit, Mich.) was used as the basal medium for antibiotic media, stock culture medium, and for master plates in replica plating (Corlett et al., 1965).

Differential and Related Media

Plates with PCAP medium as the basal medium containing antibiotics for testing different sensitivities were prepared by incorporating filter sterilized (0.22 μm millipore) antibiotics. Appropriate amounts of penicillin G(3 I.U./ml), tylosin (10 μg/ml, vancomycin (10 μg/ml) streptomycin (10 μg/ml), chloramphenicol (10μg/ml), (Sigma, St. Louis, Mo.) and a mixture of chloramphenicol (10 μg/ml) and streptomycin (10 μg/ml) were mixed individually into sterile PCAP medium (Corlett et al., 1965; Lee and Pfeifer, 1975). EMB, SS, McConkey, Staph110, Potato dextrose agar (acidified to pH 3.5), Hugh and Liefson's O/F medium (plate method), Simmon's citrate, and SIM media were prepared according to manufacturer's (Difco) instructions (Corlett et al., 1965; Lee and Pfeifer, 1975).

Oxidase Test

Oxidase test was performed on all isolates using oxidase reagent droppers (Marion Scientific).

Incubation of Prawns

Live freshwater prawns were obtained from a local supplier. The prawns were immobilized in an ice-slurry and stored aseptically on ice in an ice chest.

Isolation

Five 4-day ice-stored whole prawns were aseptically removed from iced storage, weighed, and blended separately in sterile Waring Blendors for 4 minutes at maximum speed with appropriate amounts of 0.1 percent peptone water (sterile, precooled) to obtain a tenfold dilution (blender dilution).

A 50 ml aliquot was removed from each prawn homogenate and pooled. A 1 ml sample was pipetted out of the pool and serially diluted, and 0.1 ml inocula from appropriate dilutions were spread-plated on AZOPCA plates in duplicate. After 10 days of incubation at 5°C, colonies showing proteolytic clear zones around them were picked from duplicate plates. Twenty colonies showed proteolytic activity. Inocula from each colony were streaked on a second set of AZOPCA plates for confirmation of proteolytic ability and for purification. Inocula from isolated colonies from each culture were picked up and spotted on PCAP master plates at predesignated positions (Corlett et al., 1965; Lee and Pfeifer, 1975).

Optimum Incubation Temperature

Optimum incubation temperature for use in the identification was determined by inoculating PCAP plates with the 20 cultures using the master plates for replication and incubating the replica plates at 5°C, 10°C, 15°C, 30°C, and 35°C. The temperature at which all isolates developed equally well was chosen as the optimum incubation temperature. This was found to be 15°C. All isolates grew equally well in 2-3 days at 15°C.

Identification

An identification procedure using replica plating was used (Corlett et al., 1965; Lee and Pfeifer, 1975). A PCAP master plate containing well developed colonies of each of the 20 isolates was used to inoculate a series of replica plates containing selective and differential media (antibiotic and nonantibiotic). Plates were incubated at 15°C for 2 days before interpreting the results using the identification schemes (Corlett et al., 1965; Lee and Pfeifer, 1975).

Tests for Proteolytic and Collagenolytic Activity

Culture Preparation

Identified cultures exhibiting rapid and extensive proteolysis on AZOPCA medium at 5°C were selected. Cultures were grown in 500 ml flasks containing 250 ml of tryptic soy broth. After 3 days of growth at 15°C, the culture suspension was centrifuged at $10^3 \times G$ (10,000 rpm) for 20 minutes, washed with peptone water once, recentrifuged, and suspended in 0.2 percent peptone water before using for inoculation.

Azocoll Assay for Proteolytic Activity

The procedure for proteolytic activity (Azocoll assay) provided by the Calbiochem Company was used with slight modification. As bacterial cultures were used instead of enzyme preparations, 10 ml of 0.2 percent peptone water was used to suspend the substrate (50 mg presterlized Azocoll) and the culture preparations in place of phosphate buffer. The 0.2 percent peptone water supported bacterial growth and production of proteolytic enzymes during the long incubation period at 1°C. No proteolytic activity was observed when phosphate buffer was used.

Centrifuge tubes with 50 mg of Azocoll powder each were sterilized using 25 ml propylene oxide. Ten milliliters of 0.2 percent peptone water was added to each tube and 1 ml inocula of individual bacterial culture in 0.2 percent peptone water were used to inoculate each tube. They were incubated for 4, 8, 12, and 16 days at 1°C. After incubation the tubes were centrifuged at $10^3 \times G$ (10,000 rpm) for 20 minutes, the supernatant containing the released azo dye was pipetted out and filtered (millipore 0.22 μm), and the absorbance measured at 520 nm.

Assay for Collagenolytic Activity

Fifty-milligram samples of presterilized freeze-dried prawn tissue per tube were used as the substrate. The procedure used in the Azocoll assay for sterilization and inoculation was followed. After incubating the samples for 3 and 6 days at 8°C, and 3 and 6 days at 1°C, the samples were centrifuged and the supernatant filtered. The filtered samples were assayed for hydroxyproline following the Woessner (1961) method and calculated as mg collagen

solubilized (Nip et al., 1981). The same procedure was used for the assay using presterilized insoluble bovine collagen as the substrate.

Results and Discussion

Total and Proteolytic Microflora

The total bacterial counts in freshwater prawn increased from 1.6×10^6 CFU/g at day 0 to 8.6×10^7 CFU/g at day 12 during iced storage (Table 1). This slight increase in total counts for the ice-stored prawns (*M. rosenbergii*) agreed with the Angel et al. (1981) study. It was obvious that the increase in numbers began after day 4 of iced storage and continued to increase during storage. However, the proteolytic counts did not begin to increase rapidly until after day 6 (Table 2), and they began to increase rapidly to 22 percent of the total bacterial counts by day 8, 42 percent by day 10, and to 87 percent by end of storage (day 12). This rapid increase in proteolytic microorganisms probably accounted partially for the putrid and objectionable odors coming from the prawns held in ice for more than 6 days.

Identification

The proteolytic bacteria isolated from 4-day ice-stored prawns are shown in Table 3. Five of the 20 isolates were identified as *Pseudomonas* Type I (fluorescent) based on the biochemical tests and physiological responses to differential/selective media (Corlett et al., 1965; Lee and Pfeifer, 1975).

Three isolates were identified as *Pseudomonas* Type II (nonfluorescent) (Corlett et al., 1965; Lee and Pfeifer, 1975).

Three organisms were identified as *Alteromonas (Pseudomonas) putrefaciens* as they had many characteristics in common with both *Alteromonas putrefaciens* and *Pseudomonas putrefaciens*. Since a DNA base pair ratio analysis was not done to differentiate the low G+C mol % (49 percent) containing *A. putrefaciens* from the high G+C mol % (58 percent) containing *P. putrefaciens*, it was named *Alteromonas (Pseudomonas) putrefaciens* (Parker and Levin, 1982).

Table 1.—Total aerobic psychrophilic counts (on AZOPCA medium) from fresh prawns and ice-stored prawns.

Iced storage (days)	Total aerobic psychrophilic counts[1] (CFU/g) 5°C	10°C	15°C
[2]0	1.6×10^6	1.8×10^6	4.7×10^6
4	1.6×10^6	2.1×10^6	3.0×10^6
6	5.0×10^6	3.4×10^7	1.9×10^7
8	8.8×10^6	5.6×10^7	6.6×10^7
10	5.8×10^7	8.2×10^7	8.8×10^7
12	8.6×10^7	8.6×10^7	9.0×10^7

[1]Values are averages of four trials (for fresh prawn samples) and two trials (for 4 days and 6 days ice-stored prawns). Three prawns were analyzed separately for each trial.
[2]Fresh.

Table 2.—Percentage of proteolytic microflora (on AZOPCA medium) from fresh and ice-stored prawns.

Iced storage (days)	Percentage of proteolytic colonies[1] (%) 5°C	10°C	15°C
[2]0	5.4	5.8	5.6
4	6.1	6.1	5.6
6	6.1	6.2	6.4
8	22	23	21
10	42	42	40
12	87	86.5	86.5

[1]Values are averages of three trials (for fresh prawn samples) and two trials (for 4 days and 6 days ice-stored prawns). Three prawns were analyzed separately for each trial.
[2]Fresh.

Table 3.—Identification of isolated proteolytic psychrotrophic microflora.

Isolate number	Identification
1, 5, 6, 7, 20	*Pseudomonas* group I (fluorescent)
11, 13, 16	*Pseudomonas* group II (non-fluorescent)
3, 8, 10	*Alteromonas (Pseudomonas) putrefaciens*
2, 4, 12, 14, 15, 17, 18, 19	*Flavobacterium* sp.
9	*Cytophaga* sp.

Proteolytic and Collagenolytic Activity

Four identified cultures belonging to *Pseudomonas* Group 1, *Pseudomonas* Group 2, *Alteromonas (Pseudomonas) Putrefaciens*, and a salt tolerant *Flavobacterium* exhibited rapid and extensive proteolysis on AZOPCA medium at 5°C (Table 4). At 1°C the cultures showed little activity until day 8 of incubation, after which the activity increased rapidly and reached a high level by day 12 of incubation.

Collagenolytic activity of the cultures was determined by the ability to solubilize insoluble bovine collagen (Type I, Sigma Co.) and collagen in prawn tissue. Tables 5 and 6 show the solubi-

Table 4.—Proteolytic activity of bacterial cultures at 1°C, measured as the increase in absorbance at 520 nm.

Isolate no.[1]	Absorbance[2] at 520 nm 4 days	8 days	12 days	16 days
4	0.07	0.09	0.20	0.28
5	0.19	0.20	0.25	0.35
8	0.21	0.23	0.36	0.61
16	0.20	0.21	0.26	0.38

[1]Concentrations of inocula are 2.1×10^6, 3.2×10^7, 3.8×10^7, and 4.0×10^7 cells/ml for isolates 4, 5, 16, and 8, respectively.
[2]Absorbance values are averages of four replicates.

Table 5.—Amount of hydroxyproline released from insoluble bovine collagen by proteolytic/collagenolytic bacterial cultures at various temperatures.

Isolate no.[1]	Temperature	Hydroxyproline[2] released (mg/g collagen) 3 days	6 days
4	8°C	1.50	3.70
5	8°C	4.35	6.70
8	8°C	5.55	7.60
16	8°C	3.70	7.40
4	1°C	0.75	2.85
5	1°C	3.00	6.50
8	1°C	4.70	7.60
16	1°C	3.55	7.25

[1]Concentrations of inocula are 5.2×10^6, 2.0×10^7, 3.5×10^7, and 3.8×10^7 cells/ml for isolates 4, 5, 16, and 8, respectively.
[2]Values are averages of three replications.

Table 6.—Solubilization of collagen in prawn tissues by proteolytic/collagenolytic bacterial cultures at various temperatures.

Isolate no.[1]	Temp.	Amount of collagen solubilized[2] (mg/g tissue) 3 days	6 days
4	8°C	0.103	0.208
5	8°C	0.330	0.370
8	8°C	0.363	0.435
16	8°C	0.278	0.375
4	1°C	0.055	0.160
5	1°C	0.200	0.375
8	1°C	0.253	0.445
16	1°C	0.190	0.370

[1]Concentrations of inocula are 5.2×10^6, 2.0×10^7, 3.5×10^7, and 3.8×10^7 cells/ml for isolates 4, 5, 16, and 8, respectively.
[2]Values are averages of three replicates.

lization of insoluble bovine collagen and collagen in prawn tissue by the four cultures at 1°C, and 8°C, respectively. Definite proof of collagenolytic activity was shown by the solubilization of collagen in prawn tissue and insoluble bovine collagen by the cultures at the lower incubation temperature (1°C) which is the most pertinent temperature as it is the closest to the iced-storage temperature. At higher temperatures (8°C), higher amounts of prawn collagen and insoluble bovine collagen were solubilized. The amount of solubilized insoluble bovine collagen was lower than amounts of solubilized collagen in the prawn tissue. This is possibly due to the specificity of the collagenolytic enzyme to prawn collagen and not to bovine collagen.

Proteolytic enzymes and motility are extremely important to bacteria in penetrating the muscle tissue. Gill and Penny (1977) showed that a proteolytic strain of *Pseudomonas fluorescence* penetrated a 2 cm block of meat (bull muscle) in 6 days at 5°C. The proteolytic and collagenolytic *Pseudomonas* species, *Alteromonas* (*Pseudomonas*) *putrefaciens*, and the salt tolerant *Flavobacterium* species isolated from the 4-day iced prawns were also motile. It is likely that these bacteria could have penetrated the prawn tissues and began degrading the inner muscle tissues by the production of proteolytic and collagenolytic enzymes and contributed to the mushy texture. Mushiness in the prawn tails has occurred, however, in 2-3 days of iced storage (Nip et al., 1985a). Since the collagenolytic and proteolytic bacteria did not reach a high percentage of the population until after day 6 of storage, significant bacterial degradation of tissue cannot be expected during the first 2-3 days.

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