Rapid Detection of *Pseudomonas* in Seafoods Using Protease Indicator

H. Jabbar and K. N. Joishy

ABSTRACT

The effects of *Pseudomonas* and the proteases it synthesizes in seafoods rendering them unfit for consumption are not fully recognized. A sandwich enzyme-linked immunosorbent assay (ELISA) was developed wherein protease produced by *Pseudomonas* isolated from shrimp was used as antigen, and anti-protease IgG conjugated with alkaline phosphatase was the second antibody. Purified protease and seafood samples, naturally contaminated or artificially inoculated with *Pseudomonas*, were positive by ELISA. The conventional culture method took 3 days to complete, but ELISA detected *Pseudomonas* within 24h. The rapidity, simplicity and efficacy of this test make it useful for implementation of HACCP systems.

Key Words: seafoods, *Pseudomonas*, protease, spoilage, ELISA

INTRODUCTION

MICROORGANISMS FROM THE GENUS *Pseudomonas* are a dominant group of "active spoilers" of seafoods. They can adapt to different environments while elaborating a range of enzymes that bring about the primary breakdown of the cell matrix under normal storage conditions. Such microorganisms inhabit the surface, gills and intestinal tract of fish. They are psychrotrophic and can multiply in refrigerated or frozen foods thereby reducing the shelf-life and keeping quality of products (Hobbs and Hodgkiss, 1982; Banerjee and Black, 1986). The proteases produced by *Pseudomonas* species breakdown collagen in the tissues and are important in the spoilage and secondary microbial colonization of foodstuffs, leading to changes in fish flesh. The presence and activity levels of such enzymes could therefore serve as a reliable "index of spoilage," the detection of which might serve to analyze samples long before contamination and colonization related characteristics become obvious.

Our objective was to develop a direct enzyme-linked immunosorbent assay (ELISA) for the analysis of seafood samples and determine the feasibility of using protease as an indicator for rapid detection of *Pseudomonas*. The indicator, a protease with collagenolytic activity, was obtained from a *Pseudomonas* isolate present in a frozen shrimp (*Metapeneus monocinim*) sample. The ELISA is quick, simple, reliable, and can be used to test raw material, process equipment, manufacturing environment, handlers and finished product. It could easily enable "sea-to-table" quality monitoring, as required for contemporary HACCP systems.

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Similar detection systems have been reported for *Vibrio cholerae* (Simonson and Siebeling, 1991), *Aeromonas hydrophila* (Merino et al., 1993), *Staphylococcus aureus* (Park et al., 1993), *Salmonella* species (Patel, 1994), *Listeria monocytogenes* (Curiale et al., 1996), enteropathogenic *Escherichia coli* (Chapman et al., 1997) and *Bacillus* species (Tan et al., 1997) in different kinds of foods.

MATERIALS & METHODS

Media and cultures

Seafood items (21) consisting of fresh, frozen and processed products were screened for the presence of *Pseudomonas* species, using Bacto Trypticase-soy medium (Difco) with 1% Dettol (commercial preparation by Reckitt & Colman of India, Ltd., containing 4.8% w/v chloroxylenol, 9.0% v/v terpineol, 13.1% v/v alcohol absolute denatured). A potent proteolytic isolate, *Pseudomonas*No. 421 was isolated from a frozen shrimp (*Metapeneus monocinin*) sample and identified as such on the basis of the procedures described in the *Bergey's Manual of Systematic Bacteriology* (Kreig and Holt, 1984).

Standard cultures of *Pseudomonas aeruginosa* ATCC 27853 and a *Pseudomonas aeroginosa* strain LTM isolated from a known case of infant "green diarrhea" were obtained from local hospitals for comparative studies.

Enzyme harvest and purification

The *Pseudomonas* isolate No. 421 was grown in peptone-rich medium (2% Peptone + Trypticase soy [Difco]) on a rotary shaker. The cell-free supernatant was treated with chloroform to remove pyocyanin, and the enzyme precipitated using ammonium sulphate. This enzyme preparation was dialyzed extensively against 10 mM Tris buffer (pH 8.0) and applied to a DEAE-Sepharose CL 6B (Pharmacia) column. The unbound protein was washed away and the enzyme eluted by using a stepwise NaCl gradient. The fractions were analyzed for protein content and protease activity and the active fractions were further concentrated using polyethylene glycol.

Protease activity

A qualitative assay using 0.5% gelatin in a 1% agar base with Tris buffer was used to detect protease activity. Clear zones around wells containing enzyme samples after 18h incubation at 25°C were observed on the addition of 8% TCA and measured in mm^2. The protease activity was also quantified spectrophotometrically as described by Morihara and Tsuzuki (1977), using 1% casein as substrate. One unit of protease was defined as that amount which hydrolyzed casein to produce the color equivalent to 1 μm Tyrosine/min at pH 7.4 at 37°C.

Collagenase activity

Collagen type VI prepared from rat tail (Duance, 1989) was used as substrate to estimate collagenase activity by the method described by Moore and Stein (1948). One unit enzyme released peptides from collagen equivalent in Ninhydrin color to 1 μm Leucine in 18h at pH 7.4 at 37°C in the presence of calcium ions.

The bacterial origin of the enzyme was confirmed by employing the continuous spectrophotometric rate determination assay (Van Wart and Steinbirk, 1981). One unit of enzyme hydrolyzed 1 μm N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala (FALGPA) (Sigma, product no. F-5135) per minute at 25°C in the presence of calcium ions.

All protein measurements were made by the method described by Lowry et al. (1951) using BSA as standard. The purity of the enzyme preparation was examined by polyacrylamide gel electrophoresis (Laemmli, 1970).

Antiserum production

Antiserum to the purified enzyme was

raised in two New Zealand white rabbits. The animals were inoculated with a preparation of the protease fraction emulsified with equal proportions of Freund's Complete Adjuvant (Difco). 1.2 mL of this preparation was introduced intradermally at multiple sites along the back and 0.8 mL of the same preparation injected intramuscularly in the thigh of each animal. Two booster doses were administered similarly after 3 and 8 wk respectively. Blood was collected at 10-day intervals and the antibody titre determined by Ochterlony's diffusion method. The IgG fraction was separated from serum by ammonium sulphate fractionation and its purity checked by PAGE.

Table 1—Protease activity

Sample	Protein (mg/mL)	Caseinolytic assay (U/mL)	Collagen digestion assay (U/mL)	FALGPA hydrolysis[a] (U/mL)
Culture supernates:				
Pseudomonas aeruginosa ATCC 27853	1.65	900.0	1665	1.075
Pseudomonas aeruginosa LTM	2.25	883.3	3009	1.471
Pseudomonas species No. 421	1.475	716.7	2932	0.962
Isolate 421 purified protease	0.29	560.0	1336	0.509

[a]FALGPA=N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala.

Preparation of IgG—Alkaline phosphatase conjugate

The IgG fraction was conjugated with alkaline phosphatase (an extracellular metabolite of a sea-water isolate prepared in the laboratory (results not shown), by the one-step glutaraldehyde method (Avrameas and Ternynck, 1971).

Double-antibody sandwich ELISA procedure

The working dilutions of the coating antibody and conjugate were determined using the checkerboard titration method of Voller and Bidwell (1986). Polystyrene, flat-bottomed, 96 well microtitration plates (8-well strips; Maxi-sorp, Nunc) were coated with 0.1 µg/mL of anti-protease IgG (diluted in 0.06M carbonate-bicarbonate, pH 9.6) and incubated at 4°C for 18h. The unadsorbed protein was washed away with a solution of 0.05% Tween 20 in 50 mM Tris buffer at pH 7.4. A carbonate buffer containing 1.0% BSA was used to block portions on the surface which remained unadsorbed, by adding 0.1 mL to each well and incubating for 2h at 25°C. After washing three more times, 0.05 mL/well of test samples or known positive controls were added in duplicate and incubated for 1h at 25°C. Unused coated strips were covered and stored at 4°C until further use. The test wells were washed again to remove any unbound material and 0.05 mL of the conjugate (160 µg/mL) added to each well. After 1h incubation at 25°C the washing step was repeated. Then 0.05 mL of substrate solution containing 10 µm p-nitrophenylphosphate was added to each well. The reaction was stopped after 30 min incubation at 25°C by adding 0.01 mL of 2N NaOH and the color formation was visually graded as 0, ±, 1+, 2+ and 3+ which corresponded to antigen concentrations of 0, 23, 115, 230, and 575 µg/mL respectively. Total test time was around 4h.

Conventional culture method

Varioyus fish and seafood samples collected from local retail outlets and processing units were obtained under aseptic conditions. Representative portions from each sample were transferred to two flasks containing enrichment/selective medium. One set of samples was artificially inoculated with 100 CFU/g of *P. aeruginosa* ATCC 27853. All flasks were incubated at 37°C for 18h. The debris and aggregates were allowed to settle by centrifuging at 10,000 × *g* for 20 min and the clear supernatants were tested for protease activity (by gelatin-agar and ELISA methods). Simultaneously aliquots of the medium were plated on specific media for isolation of *Pseudomonas* species. Discrete colonies suspected to be *Pseudomonas* were confirmed by cultural and biochemical tests.

RESULTS & DISCUSSION

THE PROTEASE ENZYME PRODUCED BY *Pseudomonas* species No. 421 isolated from a shrimp sample was as active as that produced by the standard *Pseudomonas aeruginosa* strain and known pathogenic isolate. Activity on native insoluble collagen was high and the specific collagenolytic activity was confirmed by the hydrolysis of FALGPA. The use of this substrate ruled out generalized collagen breakdown due to the presence of neutral proteases (Table 1).

The collagenase property of *P. aeruginosa* has been studied especially with respect to its necrotic activity during infections (Schoellmann and Fisher, 1966; Liu, 1974; Heck et al., 1986). In seafoods, proteins are present in relatively large proportions and collagen is a major constituent of bones, skin, fins, scales, connective tissue and the swim bladder. Hence, organisms producing collagenolytic enzymes can greatly affect the keeping quality of seafoods. Furthermore, psychrotrophic organisms like the *Pseudomonas* species make food unstable even after freezing. The activity and stability of protease at various temperatures needs further study. ELISA using protein-F, toxins, or whole cells as antigens are used for detection of *Pseudomonas* species in milk, dairy products and meat (Azcona et al., 1989; Gonzalez et al., 1994; Eriksson et al., 1995). In our work, protease enzyme produced by *Pseudomonas* species was used as an antigen for the development of a wholly indigenous ELISA system and applied to seafoods for the first time. The enzyme was successfully purified up to 164=fold in fraction 15 of the ion-exchange chromatography column (Fig. 1; Table 2). The SDS-PAGE analysis of this fraction showed a major band of 64,000 daltons.

The antiserum raised in two rabbits exhibited a titre of 1:32 and 1:64. Ammonium sulphate fractionation yielded IgG of 60% purity from the antiserum. This IgG fraction was successfully conjugated to the marker enzyme, alkaline phosphatase, thereby reducing the need for high cost commercial labeling enzyme preparations. Checkerboard titrations showed that the optimal concentration of IgG for coating on the wells was 0.1 µg/mL and optimum conjugate concentration was 160µg/mL. The sensitivity range of the ELISA system so developed for the detection of protease was between 23 and 575 µg/mL. Positive control samples of <23 µg/mL yielded no color reaction, whereas the color intensity decreased in samples containing >575 µg/mL. These results could be attributed to the presence of excess antigen competing for limited antibody sites on the surface of the wells, leading to fewer successful antigen-antibody bonds. A proteolytic attack on the coated IgG could also decrease the amount of bound antigen-antibody complexes, denoting a weaker signal. If this occurred, then the effect could be minimized by diluting the strongly positive samples in buffer, before adding to the wells (Olson and Ohman, 1992).

Simultaneous testing of enrichment medium supernates and *Pseudomonas aeruginosa* inoculated supernates of the eight seafood products for protease activity by different methods, demonstrated the ease and reliability of the ELISA method. All inoculated samples not only displayed a zone of clearance in the gelatin agar method and were positive by ELISA, but were also confirmed by culture method to harbor *Pseudomonas aeruginosa.* The test samples of three items were negative by all methods whereas that of the remaining five seafood items displayed protease activity by the gelatin agar method. However, of those five, the ELISA

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Table 2—Summary of purification of Isolate 421 protease

Step	Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg×10⁻³)	Recovery (%)
Cell-free supernate	200	1.35	270.0	238.87	47774	0.177	—
Dailyzed extract of ammn sulphate ppt	64	0.175	11.20	533.33	34131	3.047	71.4
Column chromatography in tris buffer:							
Fraction 13	15	0.0147	0.221	166.7	2500.5	11.30	7.33
Fraction 14	15	0.0135	0.202	200.0	3000.0	14.81	8.79
Fraction 15	15	0.0135	0.202	391.7	5875.5	29.01	17.21
Fraction 16	15	0.009	0.135	73.3	1099.5	8.14	3.22

Table 3—Detection of protease and *Pseudomonas* species in seafoods

Food	Sample[a]	Protease Zone of clearance (mm^2)	ELISA	Conventional culture method
White Pomfret, fresh	tt	0	—	Absent
	sp	324	3+	Present
Tuna, canned	tt	0	—	Absent
	sp	121	3+	Present
Ribbon fish, frozen	tt	0	—	Absent
	sp	380	2+	Present
Mackerel, frozen	tt	400	—	Absent
	sp	420	1+	Present
Black Pomfret, frozen	tt	400	—	Absent
	sp	380	1+	Present
Shrimp, PUD,[b] frozen	tt	90	—	Absent
	sp	342	1+	Present
Polynemus,[b] fresh	tt	256	2+	Present
	sp	324	2+	Present
Prawns, fresh	tt	289	3+	Present
	sp	324	3+	Present

[a]tt=test medium supernate; sp=inoculated medium supernate.
[b]PUD=peeled undeveined.

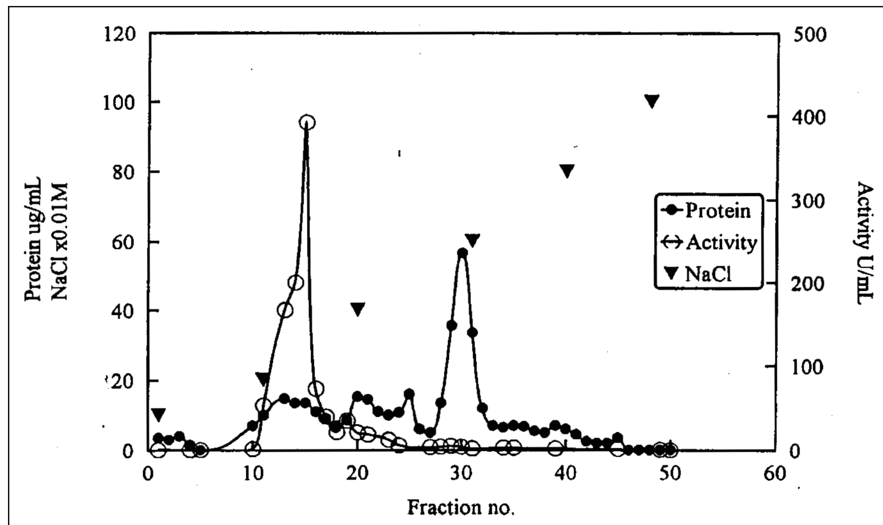

Fig. 1—Elution profile of ion-exchange chromatography for purification of protease. (DEAE-SEPHAROSE: column=23×370 mm; flow rate=2.2 mL/min; fraction=15 mL; buffer=10 mM Tris; pH 8.0; stepwise NaCl gradient elution.)

and culture methods confirmed the presence of *Pseudomonas* species in only two products, fresh *Polynemus* and frozen prawns. The zone of clearance indicated that the protease was of non-*Pseudomonas* origin (Table 3).

Results from the ELISA method were specific and obtained within 4h after enrichment, whereas culture methods took 28 to 52h. The ELISA was simple, qualitative in nature, time saving and reliable, making it feasible for offshore laboratories. However, more samples under different storage conditions need to be assessed, and scale-up studies and sensitivity need to be developed before making available a standard assay system. A color chart, for visual index could also be prepared, by which an 8-well strip could be compared and graded.

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