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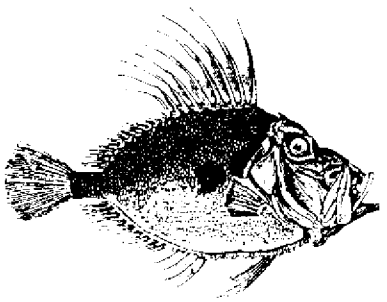

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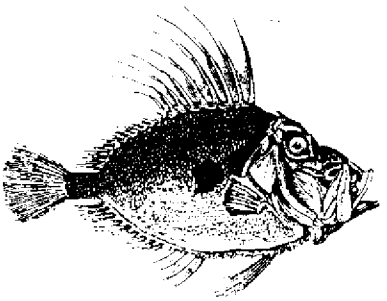

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TABLE OF CONTENTS

TABLE OF CONTENTS

(Continued, page 2 of 2)

ABSTRACTS

The following abstracts represent conference presentations not submitted as full length papers. The Tropical and Subtropical Fisheries Technological Society encourages publication of all presentations, but publication is an author's option.

PRO-PHENOLOXIDASE (PPO) FROM THE CUTICLE OF FLORIDA SPINY *LOBSTER (PANULIRUS ARGUS)*: PURIFICATION AND ACTIVATION.

M.T. Ali, M.R. Marshall, R.A. Gleeson, R.B. Shireman, and W.S. Otwell.

Department of Food Science and Human Nutrition, University of Florida, Gainesville, Fl. 32611-0163.

INTRODUCTION

The development of a dark discoloration (blackspot) on the tails of Florida spiny lobsters during iced storage is one of the major deteriorative changes which limit their shelf life. It is known to occur within two to five days on iced storage and is commonly called "melanosis" (Koburger et al, 1985). The discoloration is caused by the enzyme phenoloxidase (PO) which catalyzes the production of melanin polymers.

PO has been reported to be present in the cuticle of spiny lobsters in a latent form, pro-phenoloxidase (PPO) (Savagaon and Sreenivasan, 1978; Ferrer et al, 1989a), which goes from the latent to activated PO form by trypsin or by an endogenous enzyme with tryptic activity. However, the exact mechanism of the activation of PPO is still not fully understood.

The activation of PPO *in vivo* appears to be related to the molting cycle of the lobster. High levels of active PO have been found in lobsters ready to molt (late pre-molt), while high levels of inert PPO and low levels of active PO have been found in lobsters at intermolt and early premolt stages (Ferrer et al, 1989b). Most published work on activation of PPO has been on insects but the nature and activation of crustacean PPO have received little attention. The objective of this study was to purify PPO from the cuticle of Florida spiny lobster and examine probable mode(s) of activation of PPO.

MATERIALS AND METHODS

Florida spiny lobsters were obtained from Whitney Laboratory at Marineland, Florida. They were kept in tanks with circulating sea water. Lobster tails and cephalothorax were frozen and transported on ice to Gainesville, Florida. DL-β-3,4- dihydroxyphenylalanine (DOPA), CNBr-activated Sepharose-4B, and soybean trypsin inhibitor (SBTI) were purchased from Sigma chemical company.

PURIFICATION OF PPO.

Early pre-molt lobsters were used for the preparation of PPO. The cuticles were ground in liquid nitrogen, homogenized for 2 min., 1:4 (w/v) in 0.05M phosphate buffer (pH 7.2) (4°C), centrifuged (10,000*g*, 20 min) and the supernatant was used as a crude PPO preparation. To obtain PPO in a more purified form, the supernatant was fractionated with ammonium sulfate, and the 40-50% fraction which contained most of the PPO activity was dissolved in extraction buffer and subjected to preparative non-denaturing polyacrylamide gel electrophoresis using the method of Ferrer et al.(1989a). The gels were stained with 5mM DL-DOPA in 0.05M phosphate buffer (pH 6.5) with or without 1% trypsin, as a specific staining agent.

DETERMINATION OF PHENOLOXIDASE ACTIVITY

PO and PPO activities were determined by the method outlined by Ferrer et al (1989a).

ACTIVATION OF PPO BY HEPATOPANCREAS PROTEOLYTIC ENZYMES (HPE)

The hepatopancreas was removed from frozen lobster cephalothorax and homogenized for 1 min with 1:4 (w/v) 0.05M Tris-HCl containing 0.5M NaCl and 0.02M $CaCl_2$, pH 7.8. The homogenate was centrifuged at 10,000*g* for 20 min, filtered, and the filtrate was used for activation of PPO. Amidase activity was assayed with Benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate (Erlanger et al, 1961).
Equal volumes of HPE and PPO were mixed, incubated at room temperature, and sampled at intervals for PO activity. Concentrations of HPE and PPO were 23.6 mg/ml and 7.45 mg/ml respectively. HPE had 0.056 units of amidase activity/mg protein. Controls with HPE and PPO were used. PO activity was measured using 10mM DL-DOPA in 0.01M Tris-HCl, pH 7.0.

ACTIVATION OF PPO EXTRACT WITH CUTICULAR PROTEASE (CP)

A protease was purified from the cuticle of the lobsters using the procedure of Simpson and Haard (1984). The acetone precipitation step was eliminated and the dialyzed 40% ammonium sulfate fraction was pumped onto a CNBr-activated Sepharose 4B-SBTI affinity column.
Equal volumes of PPO extract and cuticular protease were mixed, incubated at room temperature and the mixture was sampled at intervals for PO activity. A mixture of equal volumes of PPO extract and buffer was used as a control.

ACTIVATION OF A CRUDE EXTRACT FROM CUTICLE OF EARLY PRE-MOLT LOBSTERS

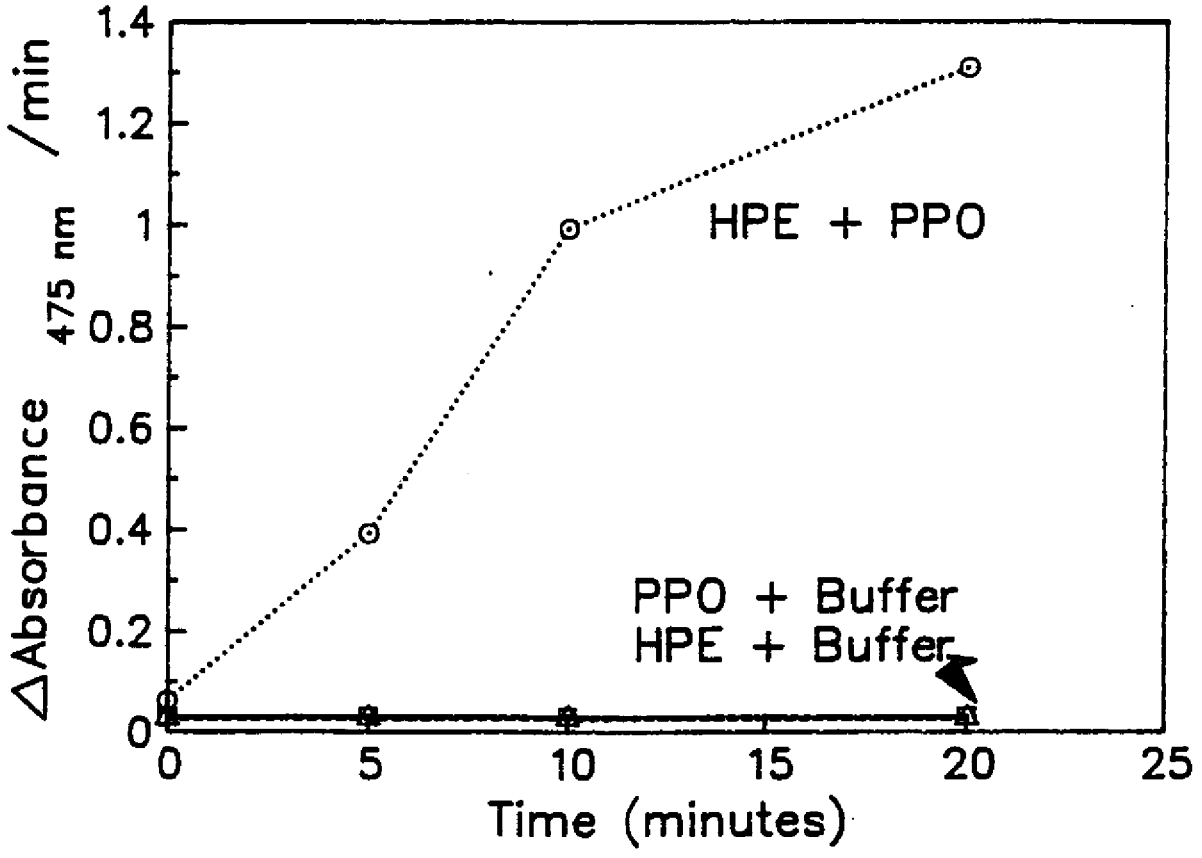
A crude extract of early pre-molt lobster cuticle was divided into two equal volumes. One portion was frozen and the other was made into several dilutions and stored at 4°C for 3 days. PO activity of the dilutions was determined on each day. After day 3, the frozen extract was thawed and combined (equal v/v) with the activated 3-day extract.

RESULTS AND DISCUSSION

A PPO form was isolated and purified by fractionation with ammonium sulfate, followed by preparative gel electrophoresis. When the gels were stained with DL-DOPA, no bands appeared. However, when 1% trypsin was added to DL-DOPA, one band which corresponded to the inert phenoloxidase (IPO2) molecule reported by Ferrer et al.(1989a) was observed.

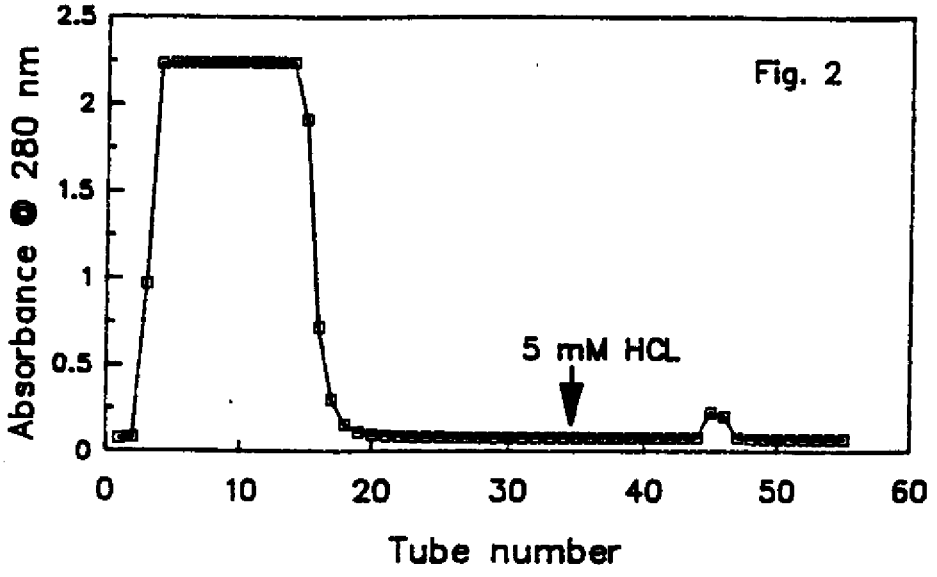
In experiments done in this laboratory, black discoloration at the top of lobster tails where the cephalothorax had been broken off, was observed after the first day of storage on ice. It was speculated that HPE enzymes may be responsible for the activation of PPO. An extract of HPE did activate PPO (Fig.1). The possibility that these digestive enzymes may activate PPO by leaching to the hyperdermal layer was examined in a separate study and sufficient evidence was not found to support this supposition. The activation by HPE appeared to occur only in those areas that are exposed to the enzymes and oxygen. The blackening on the cuticle and hyperdermal layer seemed to be an independent process and it was concluded that PPO, in the cuticle, may be activated by an endogenous proteolytic enzyme as suggested by previous investigators (Savagaon and Sreenivasan, 1978; Ferrer et al, 1989a).

An affinity chromatography column which was specific for trypsin or "trypsin-like" enzymes was used to isolate a protease fraction from an extract of the cuticle of spiny lobster (Fig.2). The yield and amidase activity (0.00356 units/mg) were very low . This fraction that eluted from the SBTI-affinity column was found to activate a crude extract of PPO (Fig.3). However, the protease could not be extracted consistently. The relationship between black discoloration and molting cycle has been confirmed by statistical analysis (Ogawa et al, 1984; Ferrer et al, 1989b). PO levels accumulate as inert forms which become activated when needed for hardening of new cuticle. It has been demonstrated that the natural activator of blood PO resides in the cuticle and

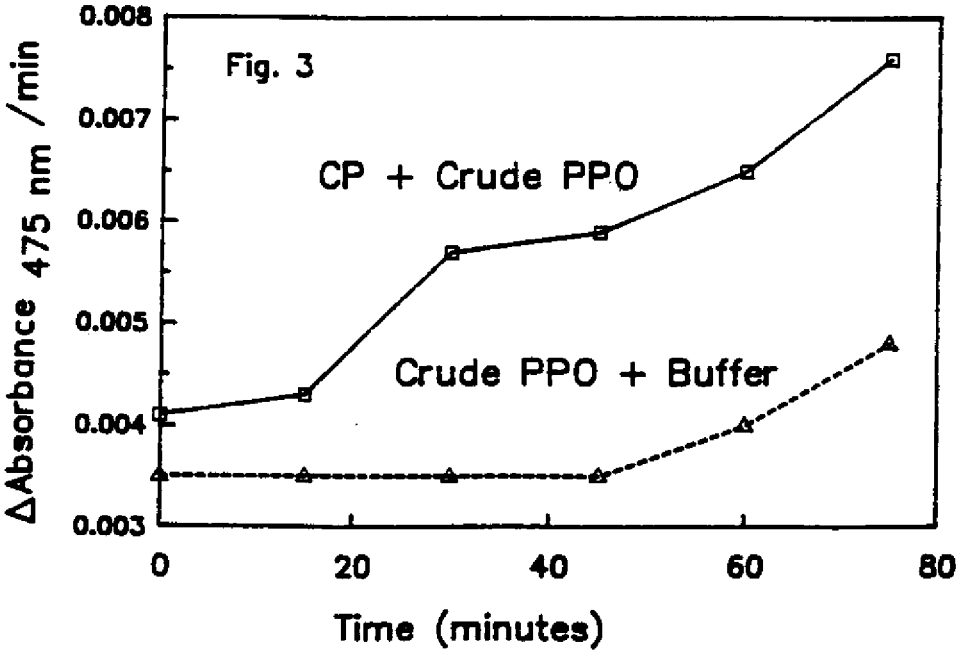


Effect of HPE on the activation of PPO

Fig. 1

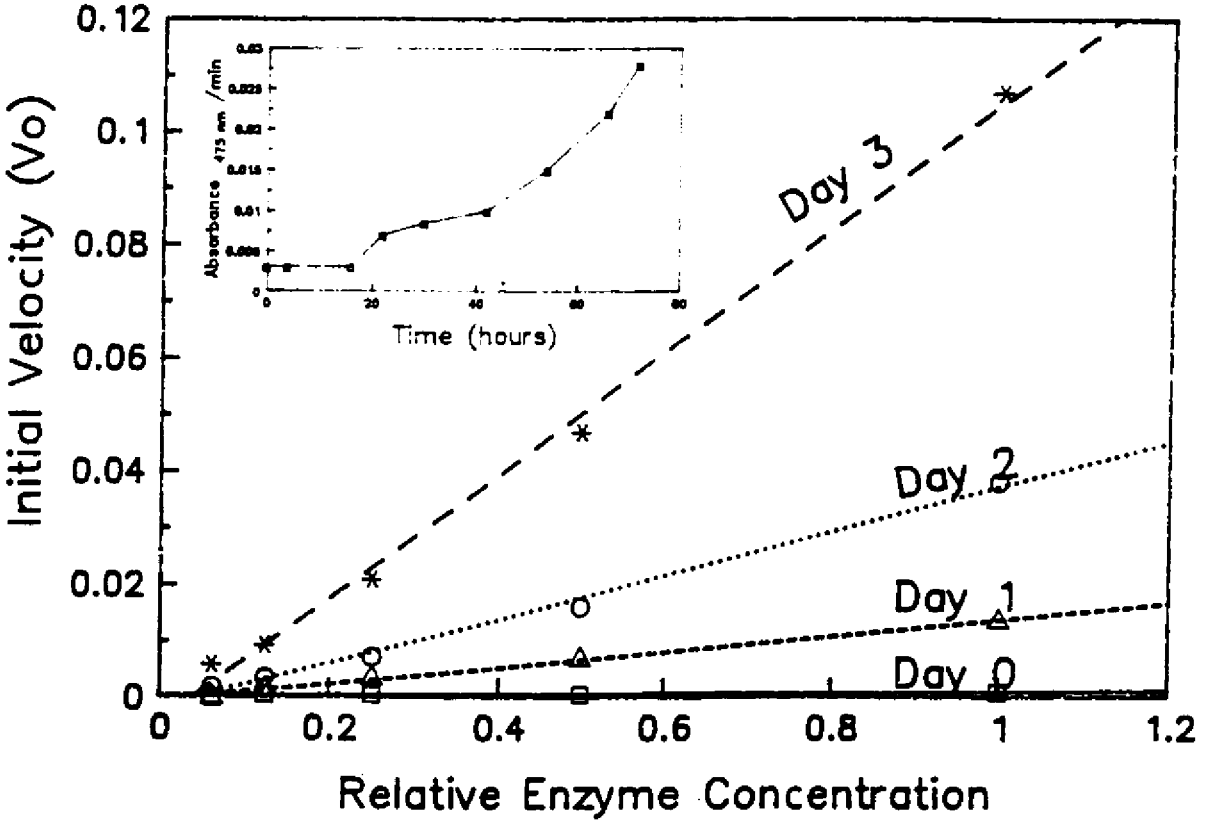


PURIFICATION OF CUTICULAR PROTEASE USING CNBr-ACTIVATED SEPHAROSE 4B-SBTI AFFINITY COLUMN



ACTIVATION OF CRUDE PPO WITH CUTICULAR PROTEASE (CP)

Fig. 4



Activity versus relative enzyme concentration during activation at 4C. Insert is a time course for activation of a crude extract at 4C.

activates PO under the influence of the molting hormone, ecdysone (Brunet, 1980). Therefore if a protease is responsible for the activation, secretion of the protease into the cuticle may be stimulated by physiological factors which are at work in the live state and controlled by biochemical mechanisms which allow induction of the enzyme when needed. It appears that factors such as molting stage and induction of the protease may be determinants in when the activator can be isolated.

Crude extracts with PPO activity, from the cuticle of Florida spiny lobster, showed a gradual increase in PO activity during storage at 4°C with the highest activity on day 3 (Ferrer et al. 1989a). The possibility that a protease may be present in the zymogen form and undergoes autocatalytic activation and then activates the inert PPO was examined. The activation of several dilutions of an extract over a three day period is shown in Fig.4.

The linearity of enzyme concentration with initial velocity showed that an inhibitor was not removed or present in the extracts. The activation process also was linear with respect to enzyme concentration and initial velocity. However, the combination of a 3-day activated extract with a frozen extract did not demonstrate direct activation that should have occurred if an activator were present.

CONCLUSIONS

PPO exists in the latent form in the cuticle of Florida spiny lobster and one form can be isolated by ammonium sulfate fractionation followed by preparative electrophoresis. Several modes of activation of PPO were examined and further work is being done in this laboratory on the mechanism of activation.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ASSAY OF TYROSINE HYDROXYLATION BY PHENOLOXIDASE FROM THE CUTICLE OF FLORIDA SPINY LOBSTER (*PANULIRUS ARGUS*).

M.T.Ali, R.S. Rolle, M.R. Marshall and C.I. Wei.

Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL. 32611-0163.

INTRODUCTION

Phenoloxidase (Tyrosinase) is the main known enzyme participating in the process of melanin biosynthesis (Penafiel et al. 1984). Crustacean integumental glands, which show cyclical activity with molting, contain a phenoloxidase (PO) active on dihydroxy phenylalanine (DOPA) believed to be involved in sclerotization (Stevenson,1985).

Phenoloxidases are copper containing proteins which are generally believed (Mason, 1956) to possess two enzymatic activities utilizing molecular oxygen: (1) monophenol hydroxylation ortho to the pre-existing hydroxyl group (cresolase activity) and (2) o-diphenol oxidation to o-quinone (catecholase activity) (Summers, 1967). The reactions following the formation of o-quinone are believed to occur spontaneously (Lerner and Fitzpatrick, 1953) and the oxidation of tyrosine proceeds through various intermediates to brownish black "melanin" pigments.

The activity of PO on diphenols is usually determined by measuring dopachrome formation spectrophotometrically at 475 nm (Fling et al. 1963) or by following the formation of melanochrome (purple pigment) at 540 nm (Vachtenheim et al. 1985). Most methods used for measuring monophenol hydroxylation are inadequate. The hydroxylation of tyrosine may be followed by measuring oxygen uptake either in a Warburg respirometer or by use of an oxygen-sensitive electrode. However, these also measure the oxygen uptake of oxidation of the o-diphenol. A more specific method is to follow the reaction spectrophotometrically at a wavelength where only the formation of o-diphenol is observed (Whitaker, 1972).

Ferrer (1987) isolated three PO forms from the cuticle of Florida spiny lobster and using a spectrophotometric method, failed to observe mono-phenoloxidase activity using tyrosine as a substrate. HPLC could potentially be used to monitor tyrosine hydroxylation and the objective of this study was

to use reversed-phase HPLC to detect formation of DOPA from tyrosine hydroxylation by PO isolated from Florida spiny lobster.

MATERIALS AND METHODS

DOPA, tyrosine and sodium octyl sulfonate were purchased from Sigma Chemical Company. Lobsters were obtained from the Florida Keys and maintained in tanks with flow-through sea water circulation at the Whitney Marine Laboratory, Marineland, Florida. Lobster tails were frozen and transported on ice from Marineland to Gainesville, FL.

PHENOLOXIDASE EXTRACTION AND PURIFICATION

The cuticle from the tails of Florida spiny lobsters were frozen in liquid nitrogen and ground to a powder in a Waring blender. The powder was stirred for 2 hours at 4°C in 0.05M potassium phosphate buffer (pH 7.2) at a ratio of 1:4 (w/v). The homogenate was centrifuged at 10,000*g* at 4°C for 20 min and the supernatant was used to purify active PO using preparative non-denaturing polyacrylamide gel electrophoresis (PAGE) at 5% acrylamide. Sample size of 1.0 mL was placed on each gel which were then run at a constant current of 10mA/tube. A 5mM DL-DOPA solution in 0.05M phosphate buffer (pH 6.5) was used as an enzymatic staining agent for PO. The PO was extracted from the gels by slicing at the Rf of PO and extracting with 0.05M phosphate buffer (pH 7.2), using a glass tissue grinder. The homogenate was filtered through Whatman #4 filter paper, and concentrated (10K Omega cell filter).

CHROMATOGRAPHIC CONDITIONS

An HPLC method using a HP 1090 chromatograph with HP-85B computer and diode array detector was used. The mobile phase was a mixture of 5% methanol in 0.1M phosphate buffer (pH 3.6) containing 0.5mM sodium octyl sulfonate. A reversed-phase 10μ C18 μ-Bondapak (Phenomenex) column 300mm length x 3.9 mm I.D. was used with a guard column 10μ C18 μ-Bondapak (Phenomenex), (30mm length x 3.9 mm I.D.). The column was kept at 40°C, flow rate was 1.0 mL/min and injection volume was 20 μl. Detection was by UV absorbance at 210 nm.

ENZYME ASSAY

0.2 ml of the enzyme (PO) extract was added to 2.8 ml 1mM tyrosine in 0.05M phosphate buffer, pH 6.5. The mixture was incubated at 25°C. Aliquots (0.5 mL) were removed at

intervals and added to 0.5 mL cold methanol, frozen, thawed and filtered through a 0.45 μm membrane and quantitated by a reversed-phase HPLC system.

RESULTS AND DISCUSSION

Monophenoloxidase activity of PO isolated from lobster cuticle has not been demonstrated before. This could be attributed to the low affinity of the enzyme for tyrosine or inadequate methods of measurement of the hydroxylation of monophenols. It has been reported (Brunet, 1980) that PO from the cuticle of insects does not present monophenoloxidase activity but, blood PO does. The diphenols needed for the cuticle PO to form the tanning quinones may be produced in the blood of the insect and transported to the cuticle. It has been suggested that a similar mechanism may occur in lobsters since both lobsters and insects use PO in the sclerotization process (Ferrer, 1987).

This present study, however, with the use of reversed phase HPLC system, demonstrates that PO isolated from the cuticle of Florida spiny lobster can catalyze the hydroxylation of tyrosine to DOPA (Figs.1 and 2). Each compound was identified by its retention time and/or by spiking with the standards under the same conditions (Fig.1). The reproducibility of retention times was very good with low standard deviations (Tables 1 and 2).

TABLE 1. Reproducibility of HPLC retention times for tyrosine separation.

Tyrosine conc. (mM)	Mean retention time (min)	Standard deviation
0.0313	7.0826	0.0616
0.0625	7.3290	0.0780
0.1250	7.7490	0.0114
0.2500	7.6830	0.0240
0.5000	7.6244	0.0109

Data are representative of the mean of 5 replicate runs.

Fig. 1

HPLC Chromatograms of Tyrosine incubated in the presence of Lobster Tyrosinase. (A) 0 Time, (B) 60 min., (C) Spiked with DL-DOPA.

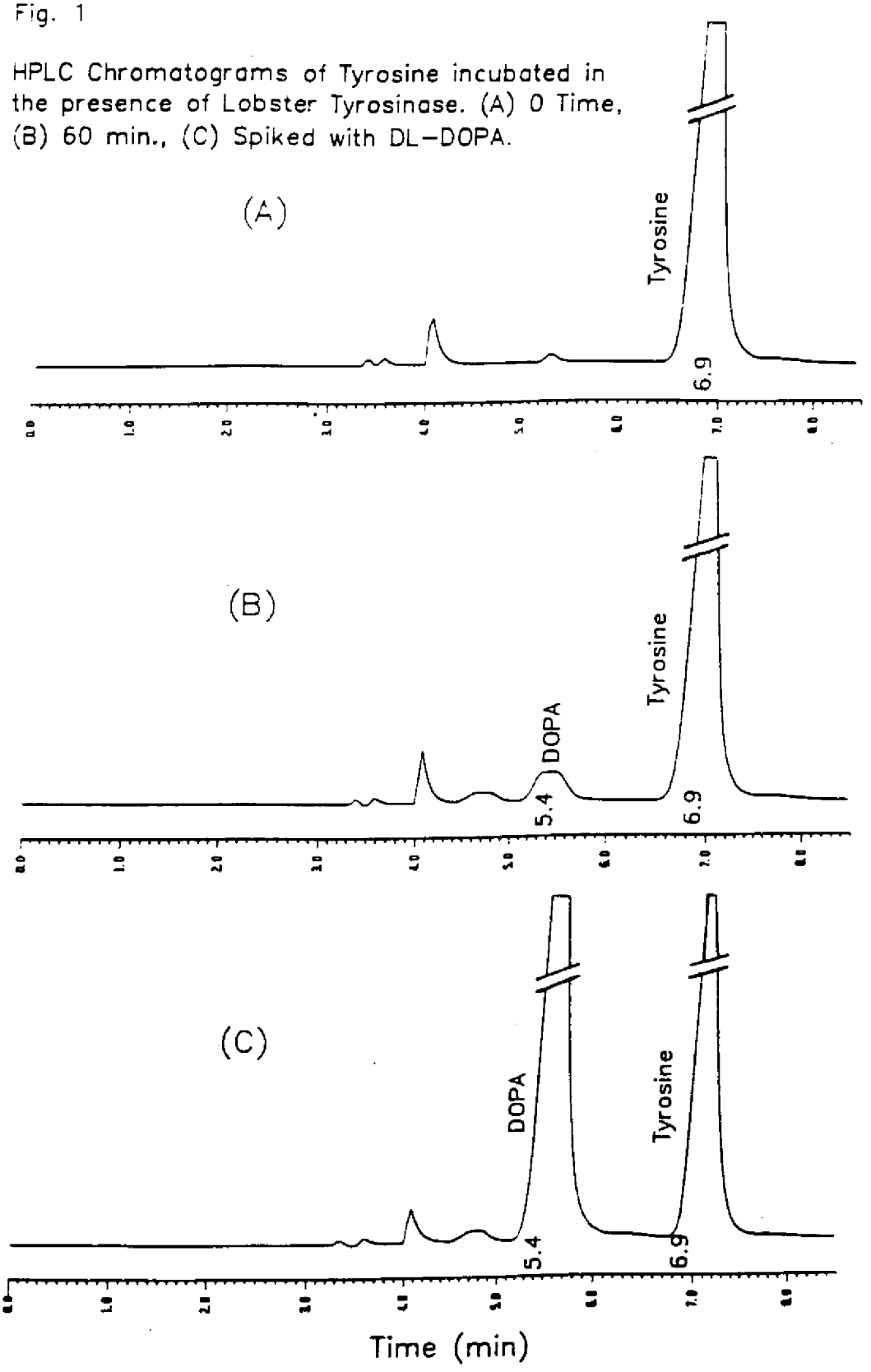

Fig. 2

TIME COURSE OF TYROSINE DISAPPEARANCE AND DOPA APPEARANCE FOR LOBSTER TYROSINASE ACTIVITY

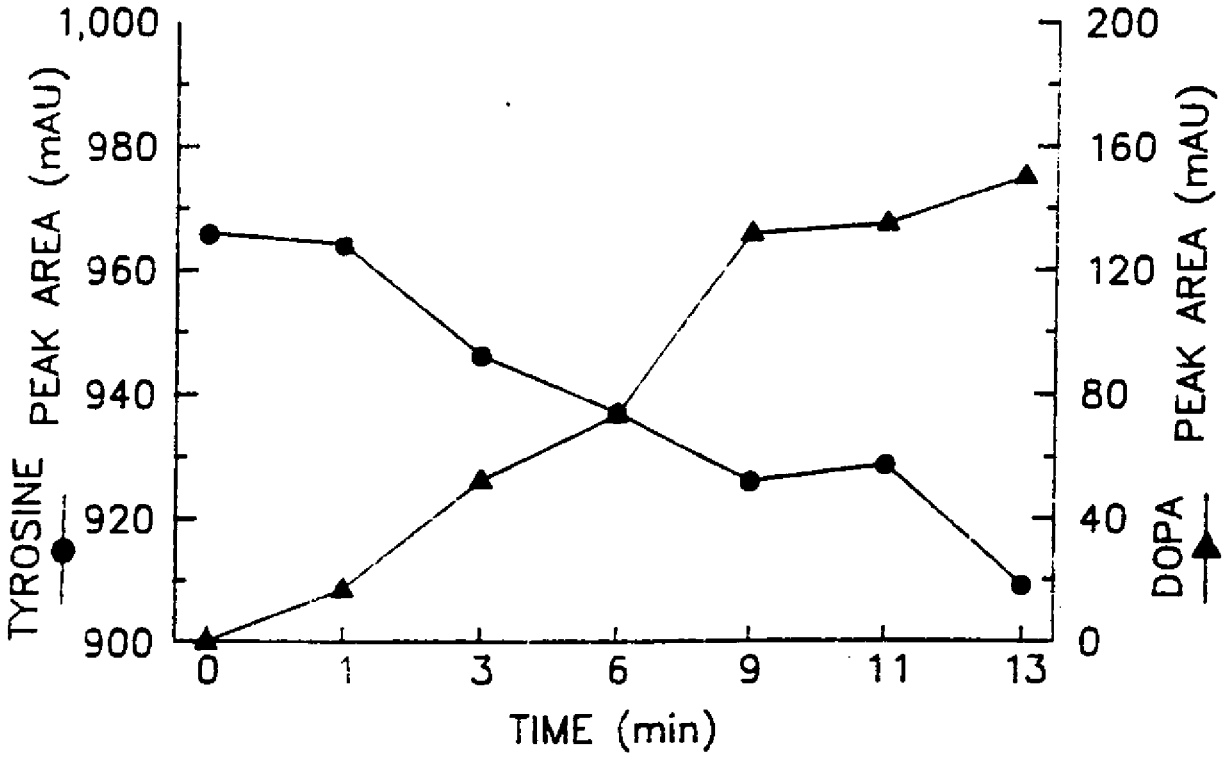

TABLE 2. Reproducibility of HPLC retention times for DOPA separation.

DOPA conc. (mM)	Mean retention time (min)	Standard deviation
0.0313	5.5696	0.0330
0.0625	5.7110	0.0448
0.1250	5.9540	0.0057
0.2500	5.9190	0.0166
0.5000	5.8880	0.0064

Data are representative of the mean of 5 replicate runs.

This paper shows preliminary investigation into the use of reversed phase HPLC as a method to facilitate the identification and quantitation of DOPA formation and may lead to a new understanding of the kinetics and mechanisms of the initial processes involved in melanin formation of crustacea.

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COMPARISON OF PHENOLOXIDASE ACTIVITY FROM FLORIDA SPINY LOBSTER AND WESTERN AUSTRALIAN LOBSTER

J. S. Chen, R. S. Rolle, M. R. Marshall, and C. I. Wei

Food Science and Human Nutrition Department,University of Florida, Gainesville, FL 32611-0163

INTRODUCTION

Polyphenoloxidase (PO) (E.C. 1.14.18.1.), also known as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, and catecholase, is widely distributed in nature (Schwimmer, 1981). PO plays an important role in the sclerotization of insects and crustaceans during the molting cycle (Andersen, 1971; Brunet, 1980; Vinayakam and Nellaiappan, 1987). However, the formation of melanins causing darkening on the surface of seafood products due to PO action is a major concern of the seafood industry. PO enzymes from various crustaceans have been characterized (Ferrer et al., 1989; Madero and Finne, 1982; Nakagawa and Nagayama, 1981; Simpson et al., 1987, 1988a). However, more information is needed especially considering the wide variation in susceptibility of crustaceans to melanosis.

A study conducted in this laboratory revealed that the Western Australian lobsters were far less susceptible to melanosis during storage at refrigeration temperature than Florida spiny lobsters. Since the water temperature of the Western Australian lobster habitat is colder than that of Florida spiny lobster, it is speculated that the PO activity for these two species could be different. Thus, the objective of this study was to compare the PO activity of these two lobster species.

MATERIALS AND METHODS

Fresh Florida spiny lobster (*Panulirus argus*) tails were obtained from Dr. R. A. Gleeson of the Whitney Marine Laboratory at Marineland, Florida. Frozen Western Australian lobster (*Panulirus cygnus*) tails were purchased from Beaver Street Foods (Jacksonville, FL); these lobsters were found to contain less than 5 ppm sulfite background residue when checked using the method of Simpson et al. (1988b).

Extraction and purification of lobster PO

After the lobster cuticle was separated from the flesh, it was frozen in liquid nitrogen and ground into a fine powder in a Waring blender. One part of the cuticle powder was added to three parts (w/v) of 0.05 M sodium phosphate buffer, pH 7.2, containing 1 M NaCl and 0.2% Brij 35. The extract was stirred for 3 hr at 4°C and the suspension was centrifuged at 8,000 x *g* (4°C) for 30 min. The supernatant was then dialyzed overnight at 4°C against 3 changes of 4 L of 0.05 M sodium phosphate buffer (pH 6.5).

Enzyme was purified using nondenaturing preparative polyacrylamide gel electrophoresis (PAGE). One-ml aliquot of crude enzyme extract was applied to

each of the eight gel tubes (1.4 cm I.D. x 12 cm length) containing 5% acrylamide gel prepared according to the method of Sigma Bulletin No. MKR-137, and run at a constant current of 10 mA/tube. PO was visualized using a specific enzyme-substrate staining method (Constantinides and Bedford, 1967); 10 mM of DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) in 0.05 M sodium phosphate buffer (pH 6.5) was used as a substrate. After the R_f value of the enzyme was determined using a sample gel, the remaining gels were sectioned at the same areas, and then homogenized in 0.05 M sodium phosphate buffer (pH 6.5) utilizing a tissue grinder. The homogenates were filtered, pooled and concentrated using an Amicon stirred cell fitted with a Pharmacia 10 K filter.

PO activity determination

Enzyme activity was determined spectrophotometrically by monitoring at 475 nm the rate of dopachrome formation from DL-DOPA (Savagaon and Sreenivasan, 1978). The assay was run at 25°C for 10 min by mixing 40 μL of enzyme extract with 560 μL of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) (Fling et al., 1963). Phenoloxidase activity was defined as μmoles dopachrome formed per min at 25°C. Unless otherwise stated, experiments were repeated at least two times.

Protein quantitation and molecular weight determination

Protein determination was performed utilizing the Bio-Rad protein assay reagent. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for molecular weight determination of the enzyme isoforms. Slab gels (16 cm x 20 cm) at 1.50 mm thickness, consisting of stacking gel (4% acrylamide) and separating gel (7.5% acrylamide), were prepared according to the Protean™ II Slab Cell Instruction Manual. Electrophoresis was carried out in a Bio-Rad Protean II Slab Cell system equipped with a Bio-Rad Model 3000/300 power supply. Constant currents of 13 mA/gel and 18 mA/gel were applied to stacking and separating gels, respectively. After enzyme samples were diluted with 4 volumes of buffer and heated at 95°C for 4 min, they were applied together with protein standards into sample wells at a protein content of 50 μg/well. A Sigma SDS-6H Molecular Weight Marker Kit containing carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa) was used. The molecular weights of the proteins were determined following the methods of Weber and Osborn (1969) and Weber et al. (1972).

pH Optima

The modified method of Gomori (1955) was followed to prepare various buffer solutions including 0.1 M sodium citrate-0.1 M HCl, pH 2.0, 3.0, and 4.0; 0.05 M sodium phosphate, pH 5.0, 6.0, 6.5, 7.0, 7.5, and 8.0; 0.1 M glycine-0.1 M NaOH, pH 9.0, and 10.0; and 0.1 M sodium phosphate-0.1 M NaOH, pH 11.0 and 12.0. The assay was performed at 25°C by adding 40 μL enzyme solution to a mixture containing 280 μL of buffer solution and an equal volume of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). Dopachrome formation was monitored spectrophotometrically for 10 min at 475 nm.

pH Stability

After enzyme mixtures containing 40 μL enzyme preparation and 120 μL of

each of the previously described buffer system were incubated at 25°C for 30 min, a 40 μL aliquot was removed and added to 560 μL of 10 mM DL-DOPA solution and the dopachrome formation was monitored.

Activation energy

Reaction mixture containing 40 μL enzyme extract and 560 μL 10 mM of DL-DOPA solution was incubated at various temperatures ranging from 20 to 60°C. The enzyme activation energy, E_a, was determined according to the Arrhenius equation by measuring the reaction rate at different temperatures and plotting the logrithmic value of V_{max} versus 1/T (Segal, 1976).

Thermostability

A 40 μL aliquot of enzyme extract was sealed in a quartz cell and incubated in a Beckman DU-7 spectrophotometer for 30 min at different temperatures ranging from 20-60°C. Following equilibration to room temperature, the enzyme extract was mixed with 560 μL of 10 mM DL-DOPA solution and then monitored for dopachrome formation.

Enzyme kinetics

The kinetic parameters (K_m and V_{max}) of the two lobster PO's were determined using the Lineweaver-Burk equation (Lineweaver-Burk, 1934). DL-DOPA and catechol solutions at concentrations varying from 1.67 to 9.92 mM in 0.05 M phosphate buffer (pH 6.5) were used as substrates. PO activity on catechol was defined as μmoles of benzoquinone formed per min at 25°C. One molecule of catechol produced one molecule of benzoquinone, which has a molar absorption coefficient (a_{m395}) of 1,350 $M^{-1}cm^{-1}$ (Whitaker, 1972).

RESULTS AND DISCUSSION

Effect of pH on PO activity and stability

In general, the PO enzymes isolated from the Western Australian lobster and Florida spiny lobster exhibited a similar pattern of sensitivity to pH changes. The Florida spiny lobster PO had a pH optimum of 6.5, which was a half unit less than that of the Western Australian lobster PO (Fig. 1). These pH optimal values for the two lobster PO were similar to that of gulf (brown) shrimp (Madero and Finne, 1982).

As to the pH-related relative activity, the Western Australian lobster PO had a broader pH range (from 5 to 9) than the Florida spiny lobster PO. The behavior of the Florida lobster PO to various pH environments observed in this study was similar to that of the IPO1 and TAPO1 forms of the Florida spiny lobster PO (Ferrer et al., 1989). For white (*Penaeus setiferus*) and pink (*Penaeus duorarum*) shrimp PO, the optimal pH environments ranged from 6-7.5 and 6.5-9.0, respectively (Simpson et al., 1988a). Aylward and Haisman (1969) proposed that the optimum pH of PO activity, which usually ranged between pH 4 and 7, varied with enzyme sources and substrates used.

The study on the effect of pH on enzyme stability revealed that the PO obtained from the two lobster species exhibited an optimum stability at pH 7

(Fig. 2). Preincubation of enzymes at pH's between 5 and 9 only slightly affected the enzyme activity when compared to that preincubated at pH 6.5. Configurational change at the enzyme active sites due to dramatic pH changes may have caused the significant decline in enzyme activity between pH 2 and 5, and between 9 and 12. Similar changes were reported to occur with the TAPO2 form of the Florida spiny lobster PO (Ferrer et al., 1989).

Effect of temperature on PO activity and stability

Most PO enzymes are heat labile; a short exposure of the enzyme to temperatures at 70-90°C is sufficient to cause a partial or total irreversible destruction of the catalytic function. The two lobster PO showed temperature-related changes of enzyme activity; enzyme activity increased with the increased temperature (Figs. 3 and 4). They also exhibited similar thermostability characteristics, although the Australian lobster PO showed a decreased activity when preincubated at temperatures greater than 30 °C. The PO from the Florida lobster showed a greater stability at a preincubation temperature of 35 °C, which was within the range of EAPO and TAPO1 forms but slightly different from the IPO1 form of the Florida spiny lobster. PO (Ferrer et al.,1989). Florida spiny lobsters are grown in warm water areas while Western Australian lobsters are found in cold water areas. These differences in environmental conditions of their natural habitats may account for the difference in the optimal thermostability between these two enzymes. PO enzymes from other crustacean sources are usually stable at temperatures ranging between 30 and 50 °C (Madero and Finne, 1982; Simpson et al., 1987, 1988a).

The activation energies, E_a, for Florida and Australian lobster PO were 6.9 and 7.5 Kcal/mole, respectively; these E_a values were similar to that of the TAPO1 form (7.8 Kcal/mole) of the Florida spiny lobster PO (Ferrer et al., 1989). However, these values are somewhat different from those of the PO prepared from white (E_a = 13.9 Kcal/mole) and pink shrimps (E_a = 11.5 Kcal/mole) (Simpson et al., 1987, 1988a).

Enzyme kinetics

Both lobster PO enzymes metabolized DL-DOPA and catechol. The Australian lobster PO displayed a relatively greater Michaelis constant (K_m = 3.57 mM) and a lower maximum velocity (V_{max} = 0.008 mM/min) with DL-DOPA than with catechol (K_m = 3.09 mM and V_{max} = 0.041 mM/min) (Fig. 5). Similar results were noted for the Florida lobster PO (Fig. 6). These two lobster PO thus had a higher affinity for catechol than for DL-DOPA. The difference in affinity for different substrate might be due to the difference in steric factors associated with the interaction between the two enzymes with the substrate. In general, K_m values vary with the enzyme sources and with the substrates. Using DL-DOPA as a substrate, Simpson et al. (1987, 1988a) showed that pink shrimp PO had a lower Michaelis constant of 1.6 mM than white shrimp PO of 2.8 mM.

The Australian lobster PO exhibited a higher affinity for DL-DOPA and catechol than the Florida lobster PO. However, the latter showed a much greater rate in the metabolism of DL-DOPA or catechol than the former. Such phenomena can probably be interpreted based on the difference in enzyme turnover number on the substrates, i.e., the molecular activity or molar activity, which is defined as "the number of moles of substrate transformed per minute per mole of enzyme under the optimum conditions" (Segal, 1976). The data in Table 1

Table 1. Comparison of phenoloxidase (PO) activity between Florida spiny lobster and Western Australian lobster

PO	Specific Activity[a] (ΔA/min·mg protein)	Turnover Number[a] (Units/mole enzyme)	$V_{max}/2K_m$ $(min^{-1})^b$		V_{max}/K_m $(min^{-1})^c$	
			DL-DOPA	Catechol	DL-DOPA	Catechol
Florida spiny lobster	0.36	5.7×10^7	0.024	0.079	0.048	0.157
Western Australian lobster	0.03	4.7×10^6	1.1×10^{-3}	6.6×10^{-3}	2.2×10^{-3}	1.3×10^{-2}

[a]DL-DOPA was used as a substrate.

[b]Substrate specificity.

[c]Physiological efficiency.

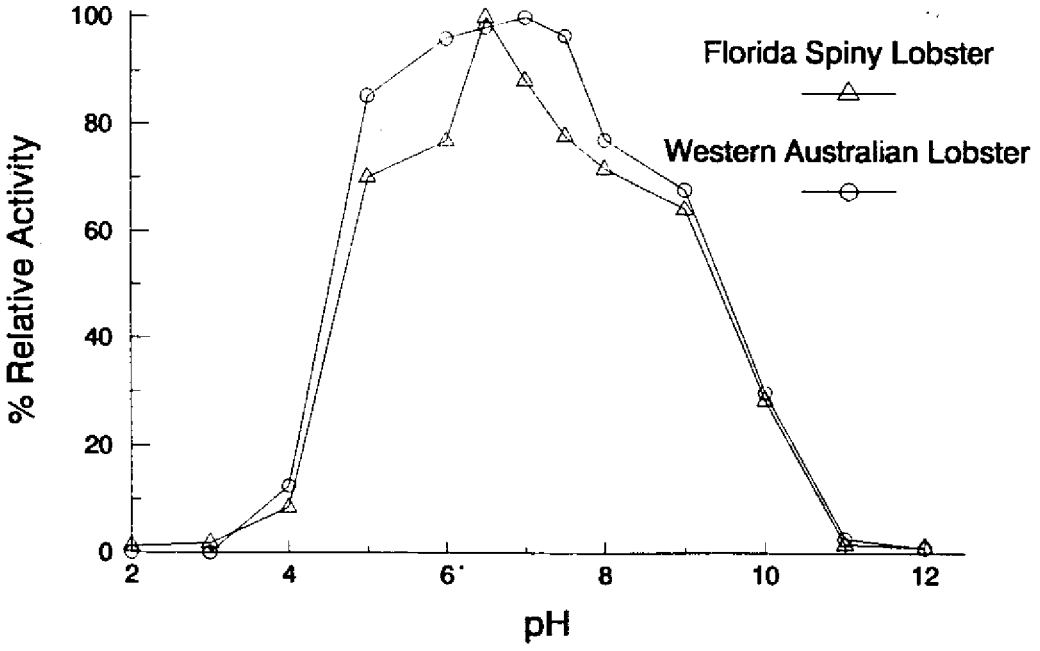

Figure 1. The effect of pH on the activity of phenoloxidase obtained from Florida spiny lobster (Δ) and Western Australian lobster (o).

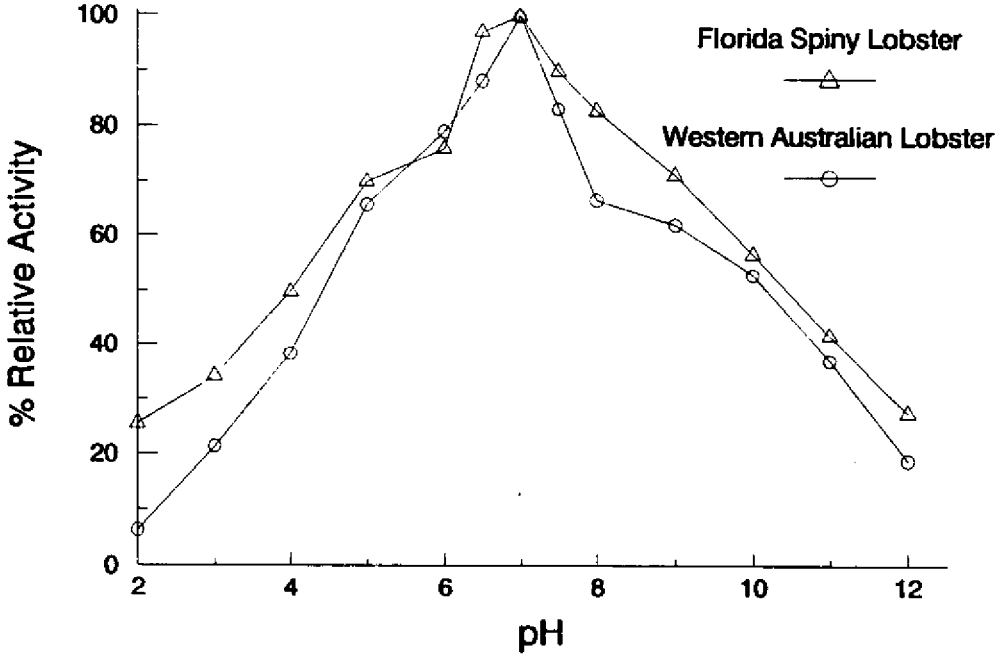

Figure 2. The effect of pH on the stability of phenoloxidase obtained from Florida spiny lobster (Δ) and Western Australian lobster (o).

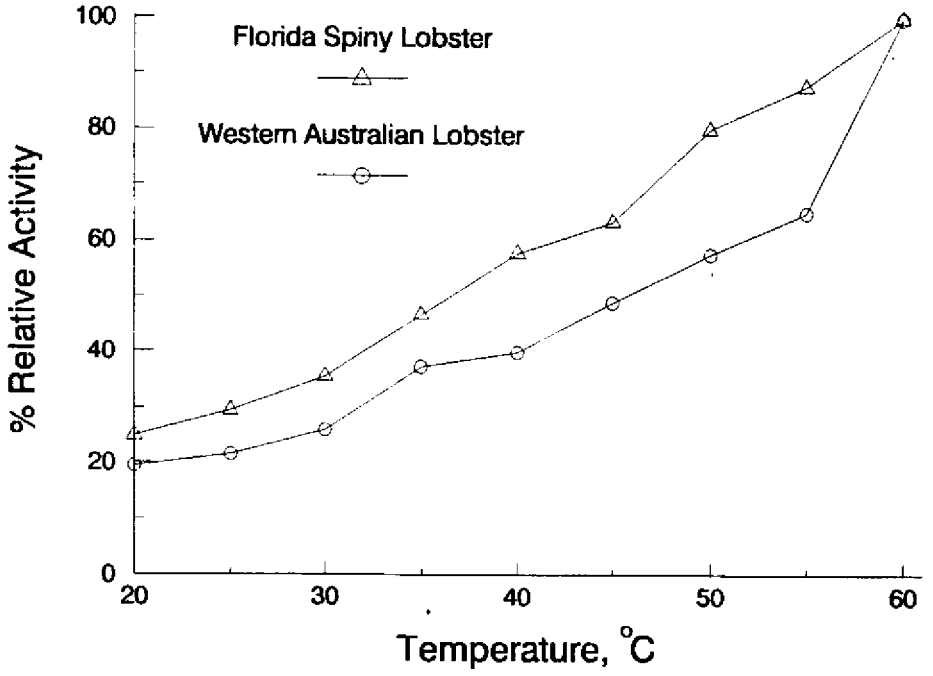

Figure 3. The effect of temperature on the activity of phenoloxidase obtained from Florida spiny lobster (Δ) and Western Australian lobster (o).

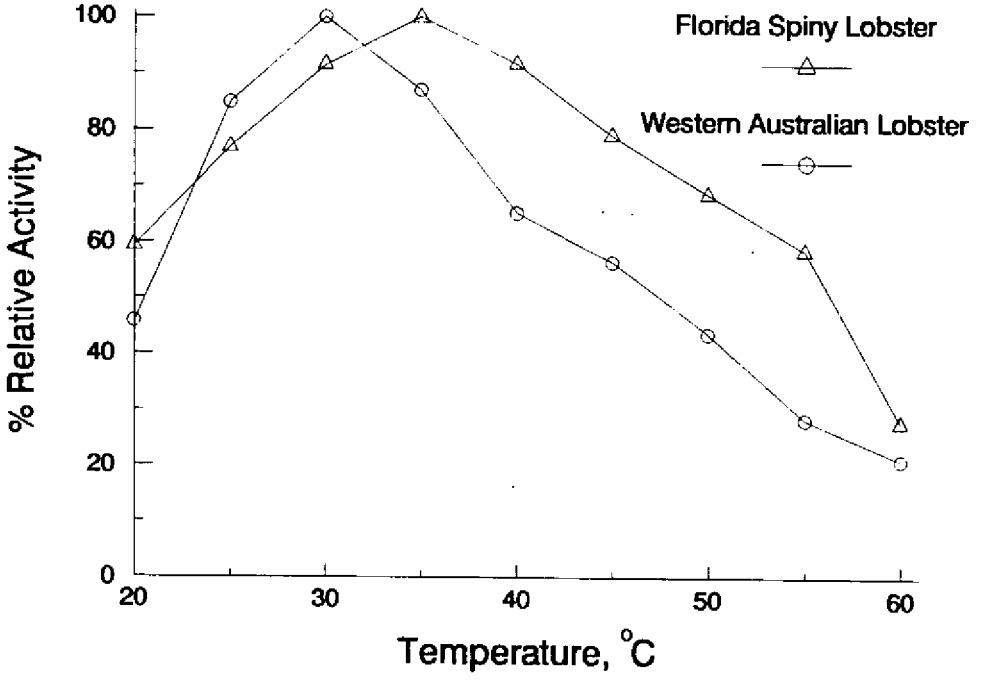

Figure 4. The effect of temperature on the stability of phenoloxidase obtained from Florida spiny lobster (Δ) and Western Australian lobster (o).

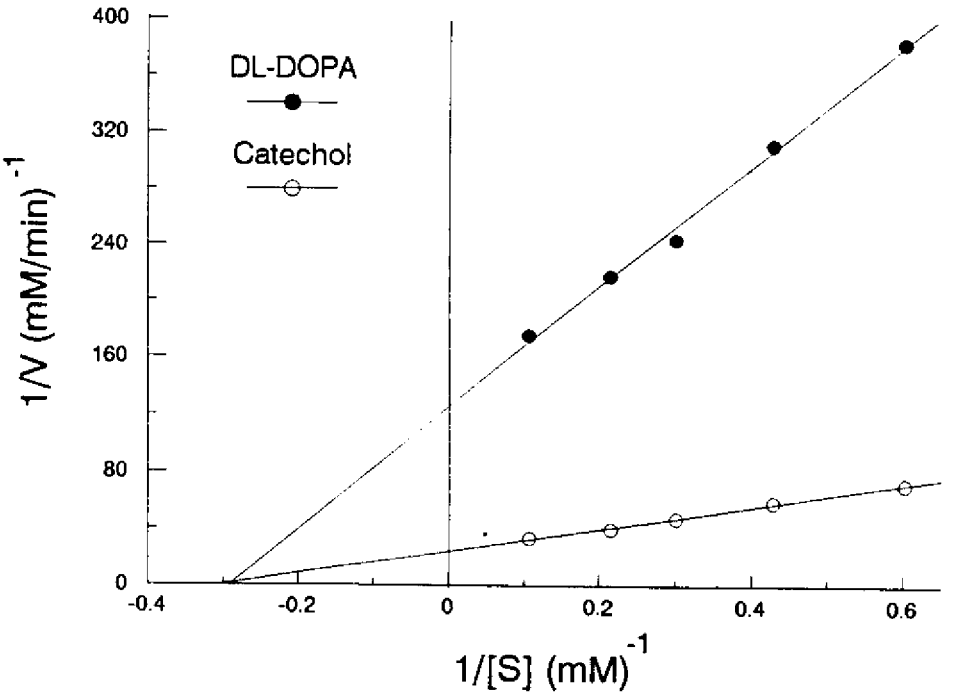

Figure 5. Double reciprocal plot of DL-DOPA and catechol metabolism by phenoloxidase isolated from Western Australian lobster. The K_m of DL-DOPA and catechol were determined to be 3.57 and 3.09 mM, respectively; the V_{max} determined to be 0.008 and 0.041 mM/min, respectively.

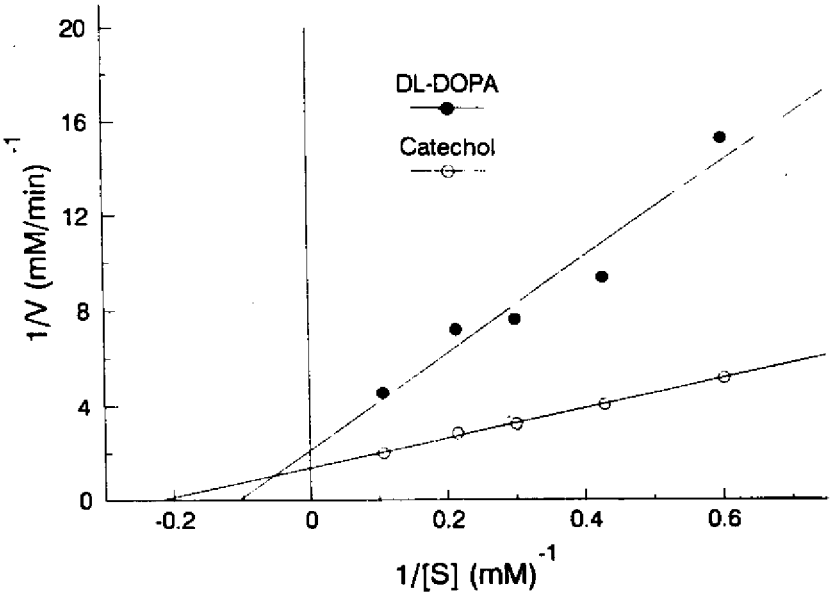

Figure 6. Double reciprocal plot of DL-DOPA and catechol metabolism by phenoloxidase isolated from Florida spiny lobster. The K_m of DL-DOPA and catechol were determined to be 9.85 and 4.58 mM, respectively; the V_{max} determined to be 0.48 and 0.72 mM/min, respectively.

indicated that the Florida lobster PO not only showed a higher specific activity but also had a greater turnover number on the substrate than the Australian lobster PO. In terms of substrate specificity ($V_{max}/2K_m$) (Lavollay et al., 1963) or physiological efficiency (V_{max}/K_m) (Pollock, 1965), the Florida lobster PO also showed a higher efficiency on DL-DOPA and catechol than the Australian lobster PO (Table 1).

Molecular weight and isoform determination

Figure 7 shows the SDS-PAGE pattern of the two lobster PO enzymes; they both had two isoforms. The molecular weights of the Florida lobster PO isoforms were determined to be 90 and 100 kDa, while those of the Australian lobster were 93 and 95.5 kDa. The molecular weight of these isoforms were higher than those of white shrimp (30 kDa), and pink shrimp (40 kDa) (Simpson et al., 1987, 1988a), but lower than that of brown shrimp (210 kDa) (Madero and Finne, 1982). The similar molecular weights suggest that PO from Florida spiny and Western Australian lobsters have similar structures. Also, although these two animals were grown in two different water areas, a recent study using immunological techniques with rabbit antisera against the Florida spiny lobster PO revealed that these two lobster PO's shared cross-reactivity (Rolle et al., 1989).

SUMMARY

The phenoloxidase isolated from the Western Australian lobster and Florida spiny lobster showed very similar patterns in their response to the effect of pH and temperature on enzyme activity and in SDS-PAGE profile. Using DL-DOPA and catechol as substrates, the former enzyme was shown to have a higher affinity but lower efficiency (specificity) than the latter. These results thus indirectly indicate that these two PO enzymes are very similar in conformational structure, however, they show distinctly different properties for catalyzing phenolic substrates. This may explain the differences in susceptibility of Florida spiny lobster to melanosis compared to Western Australian lobster.

ACKNOWLEDGEMENTS

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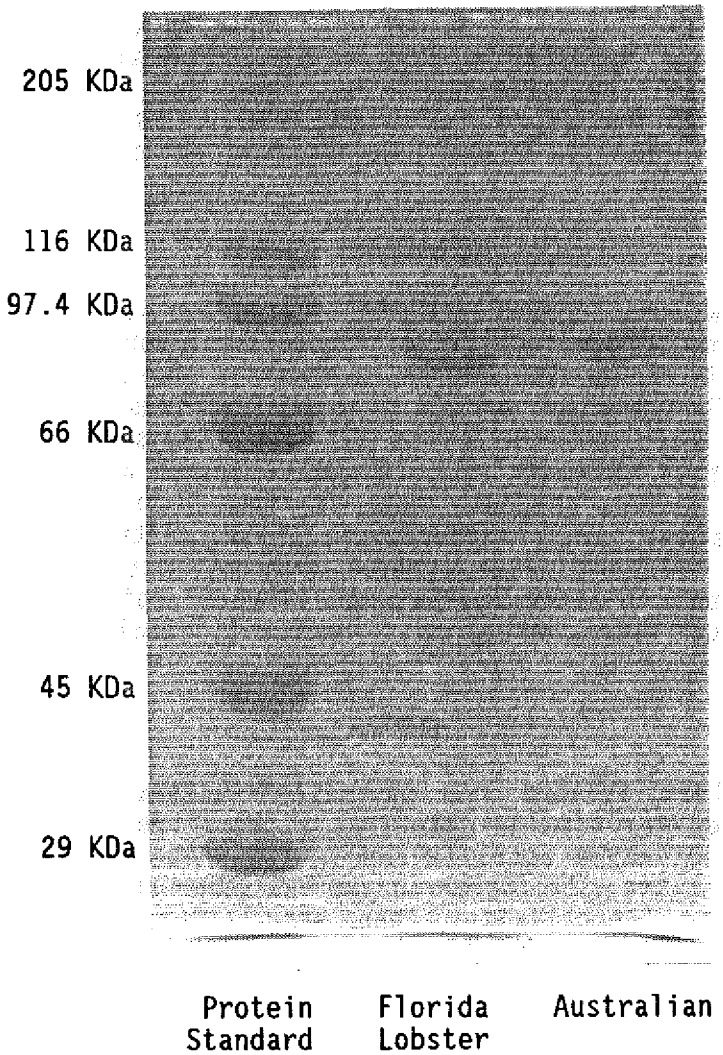

Figure 7. SDS-PAGE pattern of phenoloxidase from Florida spiny lobster and Western Australian lobster. Protein standards are also included. The numerical designations represent the molecular weight of the protein bands.

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IMMUNOLOGICAL CHARACTERISTICS OF PHENOLOXIDASE ANTIBODIES

R.S. Rolle, M.R. Marshall, C.I. Wei and J.S. Chen

Food Science and Human Nutrition Department
University of Florida Gainesville, FL 32611

INTRODUCTION

Phenoloxidase enzymes exist in crustaceans in a pro-enzyme form designated prophenoloxidase. Prophenoloxidase is inert, and must undergo activation in order to effect melanosis or "black spot", a defect which connotes spoilage of seafood products to the consumer.

Activation of prophenoloxidase in crustaceans is well documented (Soderhall and Unestam, 1979; Soderhall and Hall, 1984; Marshall et al., 1984; Savagaon and Sreenivasan, 1978; Ferrer et al., 1989). It has been reported to occur via *in vitro* treatment with carbohydrates, purified fungal glycoproteins and laminarin pentose (Soderhall and Unestam, 1979) and proteolytic enzymes (Savagaon and Sreenivasan, 1978; Marshall et al., 1984; Ferrer et al., 1989). *In vivo* activation of prophenoloxidase in lobsters and shrimp by a tryptic-like enzyme in tissues of these crustaceans has also been reported (Savagaon and Sreenivasan, 1978).

A prophenoloxidase enzyme (IPO1) was isolated from the Florida spiny lobster (Ferrer et al., 1989). This enzyme underwent "endogenous" activation upon incubation at $4^{0}C$ for three days, to yield an active phenoloxidase form (EAPO), having a molecular weight of 62.5 KDa. *In vitro* trypsin treatment of IPO1 also yielded an active phenoloxidase form (TAPO) having a molecular weight of 64 KDa. These two active phenoloxidase forms showed differences with respect to kinetic parameters, pH and temperature properties (Ferrer et al., 1989).

Katz and Mayor (1969) observed a similar phenomenon in that *in vitro* trypsin treatment of sugar beet phenoloxidases resulted in changes in kinetic parameters of these enzymes. The proteolytic activation of phenoloxidases may very well involve conformational changes which result in alterations in their kinetic parameters (King and Flurkey, 1987).

The present report employed the use of immunological techniques to determine whether EAPO and TAPO species described by Ferrer et al.(1989) are indeed structurally different phenoloxidase forms.

MATERIALS AND METHODS

Preparation of phenoloxidase

Preparation of lobster cuticle

The cuticle was removed from the tail of the Florida spiny lobster (*Panulirus argus*). It was subsequently frozen in liquid nitrogen and homogenized to a fine powder in a Waring Blender. The cuticle powder was then stored at -15^{0}C and utilized as required.

Preparation of EAPO and TAPO

Crude phenoloxidase cuticular extracts were prepared from the cuticle powder according to the method of Ferrer et al. (1989).

Endogenously activated phenoloxidase extracts (EAPO) in 0.05M potassium phosphate buffer were prepared by allowing the crude cuticular extracts to sit at 4^{0}C for three days. They were subsequently frozen and stored at -15^{0}C for further purification via polyacrylamide gel electrophoresis (PAGE).

Trypsin activated phenoloxidase extracts (TAPO) were prepared by adding an aliquot of 0.1% trypsin solution in water (0.1mL/mL crude cuticular extract) to the crude phenoloxidase cuticular extract immediately prior to further purification via PAGE.

Purification of EAPO and TAPO

PAGE gels (5%) were prepared according to Sigma Bulletin #MKR-137 (1984). An aliquot (1mL) of either TAPO or EAPO was loaded onto each of the 8 preparative gels (1.4cm wide x 12cm long) and electrophoresis of samples was conducted at a constant current of 8ma/gel tube. Following electrophoresis, a sample gel was stained in 10mM D,L-β-3,4-dihydroxyphenylalanine (DOPA) dissolved in 0.1M potassium phosphate buffer pH 6.5 and the R_f of phenoloxidase was determined. Other gels were subsequently marked and sliced at the determined R_f.

Phenoloxidase was extracted from the sectioned gels by homogenizing in 0.05M potassium phosphate buffer (pH 7.2) utilizing a Potter Elvejheim homogenizer. The gel homogenate was filtered through a Whatman #1 filter paper and subsequently washed till the gel residue showed very little to no phenoloxidase activity. The phenoloxidase-containing filtrate was concentrated via ultrafiltration utilizing a 50K Omegacell filter (Filtron, Pharmacia Chemical Co.). The concentrated enzyme was further subjected to preparative gel electrophoresis on 7.5% PAGE gels prepared according to Sigma Bulletin #MKR-137, (1984) and treated as described. The

concentrated extracts which constituted the purified phenoloxidase enzyme were frozen at -15°C and utilized as required.

Immunization schedule

A 2mL aliquot of antigen solution (purified EAPO or TAPO) in 0.9% saline solution was emulsified with MPL + TDM adjuvant (Ribi Immunochemical Research Inc.). Three New Zealand female rabbits were used for immunization with each antigen. The rabbits were intramuscularly injected with antigen in the thigh (0.5mL/injection site) at intervals of three weeks over a five-month period. They were bled through the marginal ear vein; the blood was retained at room temperature for two hours and then refrigerated overnight at 4°C. Antisera were collected by centrifuging the blood at 5000 x g for 5 min. The antisera were stored at -20°C and screened for affinity and titer by the Ouchterlony Immunodiffusion technique as outlined by Hudson and Hay (1972) with a modified destaining reagent which constituted 40% methanol, 7% acetic and 53% water.

EAPO and TAPO antisera pretreatment and standardization

The protein content of EAPO and TAPO was adjusted to 13.6mg/mL by dilution with 0.05M potassium phosphate buffer, pH 6.5. A 1mL aliquot of the standardized antisera thus obtained was diluted via serial dilution in the same buffer to yield 1/8, 1/16 and 1/32 parts antisera/mL. The diluted antisera were mixed in a ratio of 1:1 (v/v) with either 0.026 units of TAPO or 0.029 units of EAPO. Controls were run by pre-incubating either TAPO or EAPO in 0.05M potassium phosphate buffer, pH 6.5. The phenoloxidase-antisera mixtures along with the controls were incubated at 4°C overnight and centrifuged at 8000 x g for 5 min. Residual phenoloxidase activity in the supernatants thus obtained was assayed. No residual phenoloxidase activity was detected in sediments.

The percentage of enzyme bound was computed as follows:

$$\% \text{ Enzyme bound} = \frac{\text{CTRL activity} - \text{Sup activity} \times 100}{\text{CTRL activity}}$$

Where:

CTRL activity = Activity of phenoloxidase enzyme in 0.05M sodium phosphate buffer, pH 6.5.

Sup activity = Residual phenoloxidase activity in the supernatants of pre-incubated phenoloxidase antiserum mixtures.

Basic enzyme assay

A 70 μl aliquot of the supernatant fraction obtained as described above was added to 930 μl of 10mM DOPA dissolved in 0.1M potassium phosphate buffer pH 6.5. $\Delta A_{475nm/min}$ was recorded at 25°C over 10 min.

RESULTS AND DISCUSSION

Antisera against EAPO (S) and TAPO (T) exhibited a relatively low titer when tested against their respective antigens (EAPO and TAPO) or when allowed to cross-react (Figs. 1 and 2). A consistently low titer was obtained despite the use of adjuvant for immunization and repetitive boosters to the rabbits over the 5-month period.

Cross-reactivity studies were conducted to determine antiserum specificity utilizing the Ouchterlony immunodiffusion technique. S and T antisera (4μL/well) were tested against TAPO and EAPO (4μL/well)(Figure 3). A single immunoprecipitin band was observed when the S and T antisera were tested against their respective antigens or against the antigens of each other (i.e., S vs. TAPO and T vs. EAPO). Further, when S and T antisera incubated in adjacent wells were allowed to react with a single antigen (either EAPO or TAPO), the immunoprecipitin pattern produced constituted a fused continuous arc, with the absence of spurs at the intersections of the precipitin bands, indicating that the EAPO and TAPO enzymes share identical antigenic sites.

S and T antisera obtained from all three rabbits immunized were further tested against a TAPO enzyme (TAPO(A)) isolated from a West Australian lobster species (*Panulirus cygnus*) (Fig. 4). A single immunoprecipitin band was observed in all cases for S antisera tested against TAPO(A), and T antisera tested against TAPO(A). Cross-reactivity evident in this situation would thus indicate that the TAPO(A) enzyme shares common antigenic sites with both TAPO and EAPO of the Florida spiny lobster. Control antisera from rabbits that were not immunized, on the other hand did not show any immunoprecipitation reaction with TAPO(A).

Double reciprocal plots of residual activity of TAPO and EAPO subsequent to their pre-incubation in the presence of various amounts of S and T antisera followed Michaelis-Menten kinetics (Figs. 5-8). Phenoloxidase catalysis of DOPA metabolism was observed to undergo linear non-competitive inhibition in the presence of S and T antisera. A linear non-competitive inhibitor is one which binds to an enzyme at a site other than its active site (Whitaker, 1972). The results thus indicate that the antigenic site

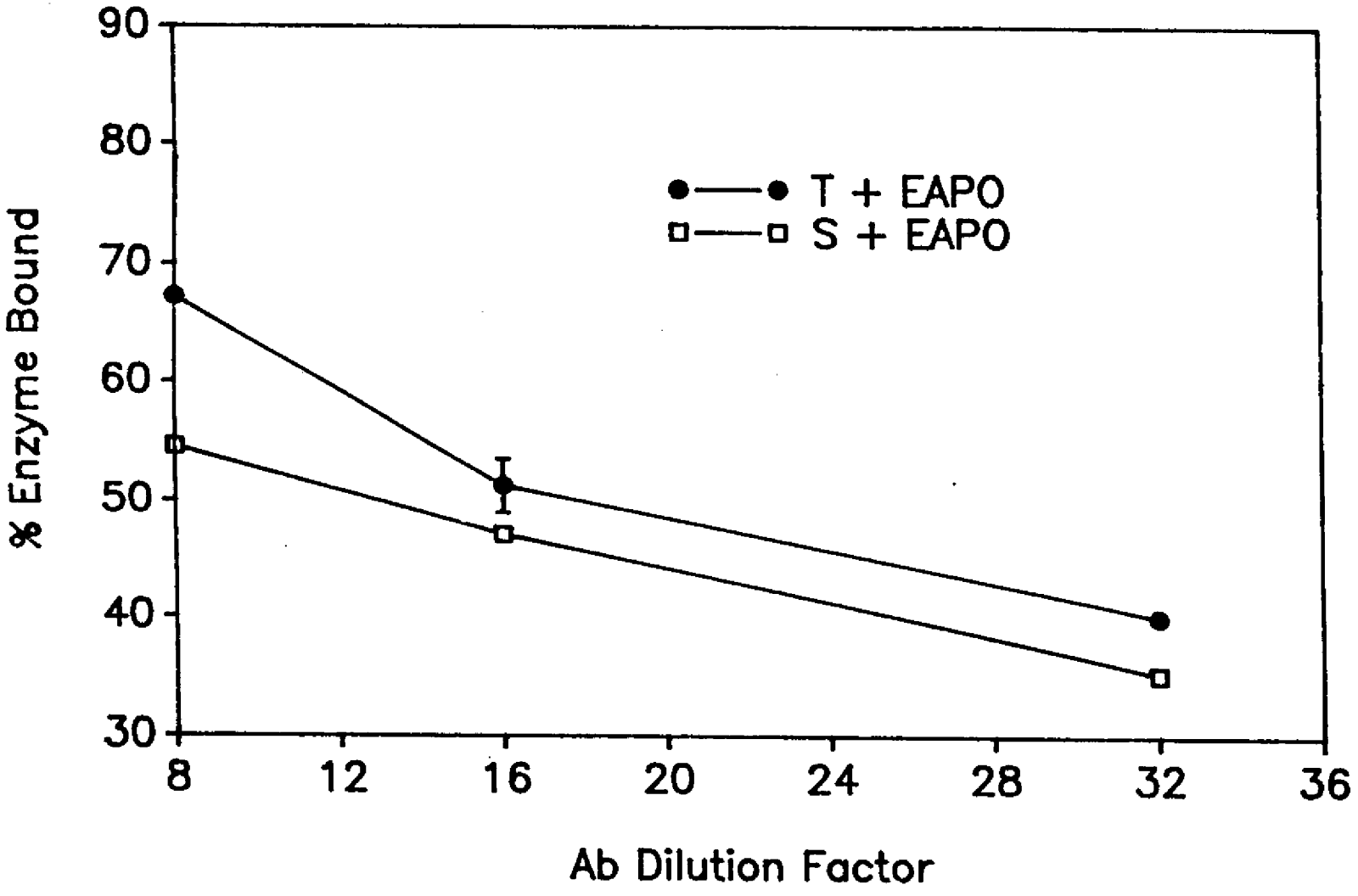

FIGURE 1. ENZYME BINDING CAPABILITY OF THE VARIOUS ANTISERA AT DIFFERENT LEVELS AFTER PRE-INCUBATION WITH 0.029 UNITS OF EAPO ACTIVITY.
ASSAY CONDITIONS WERE AS DESCRIBED IN THE METHODS.

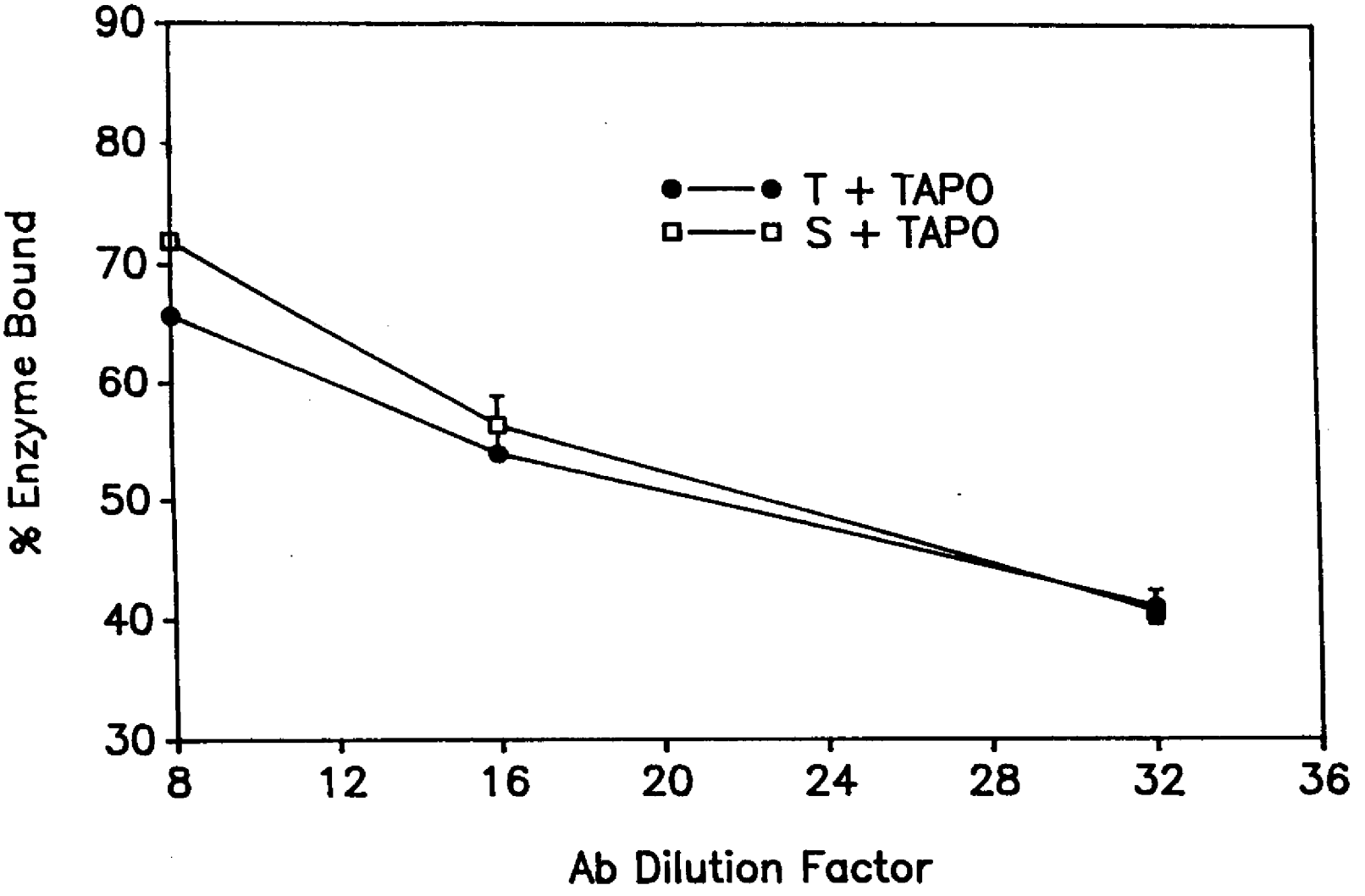

FIGURE 2. ENZYME BINDING CAPABILITY OF THE VARIOUS ANTISERA AT DIFFERENT LEVELS AFTER PRE-INCUBATION WITH 0.026 UNITS OF TAPO ACTIVITY.
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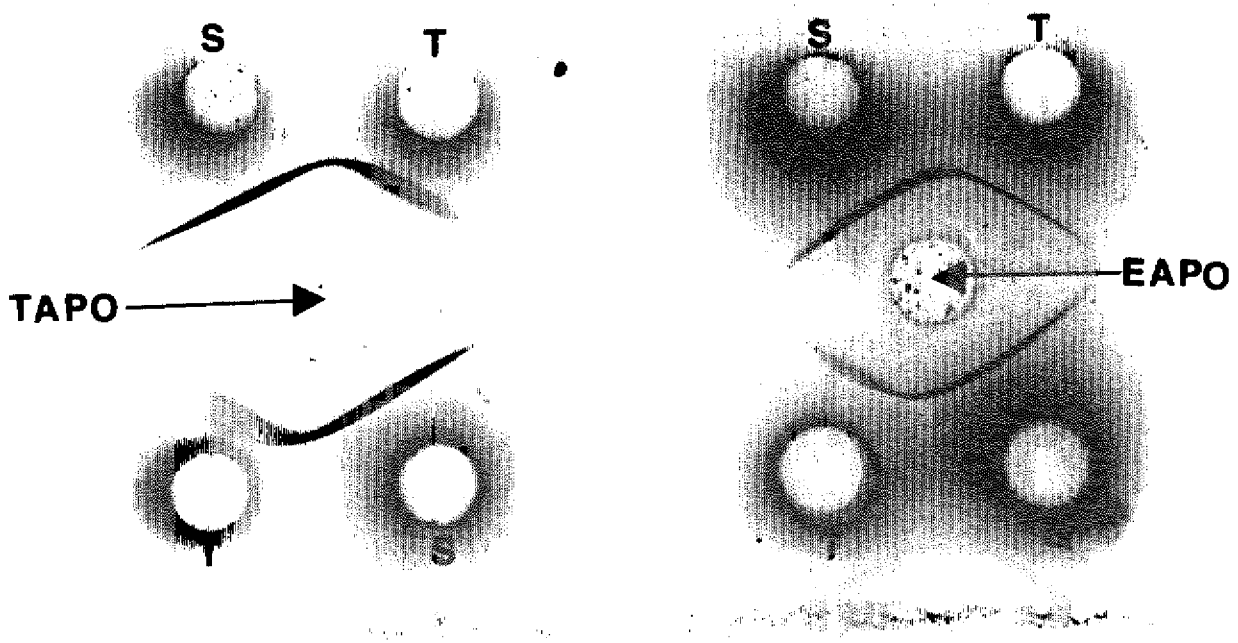

FIGURE 3. PRECIPITIN BANDS OBSERVED WHEN ANTISERA AGAINST EAPO (S) AND TAPO (T) WERE ALLOWED TO DIFFUSE TOWARD BOTH EAPO AND TAPO.

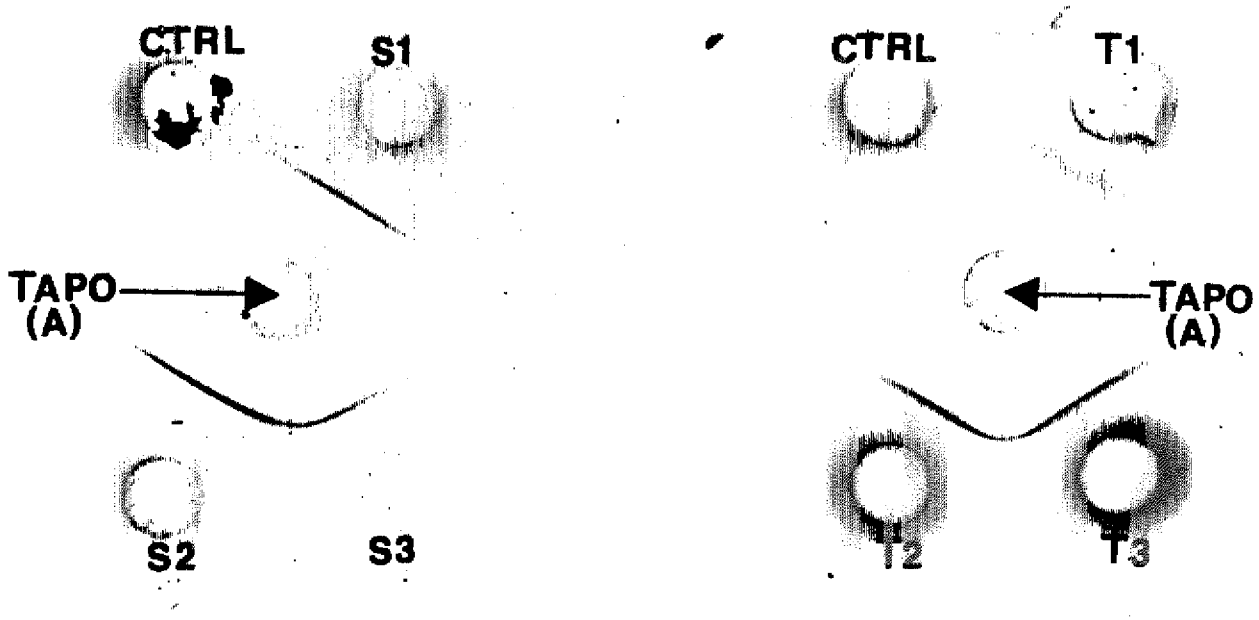

FIGURE 4. PRECIPITIN BANDS OBSERVED WHEN ANTISERA AGAINST EAPO (S1, S2, S3) AND TAPO (T1, T2, T3) ISOLATED FROM FLORIDA SPINY LOBSTER WERE ALLOWED TO DIFFUSE TOWARD A TAPO ENZYME (TAPO(A)) ISOLATED FROM AN AUSTRALIAN LOBSTER SPECIES. THE CONTROL WELL (CTRL) CONTAINED SERUM FROM A RABBIT WHICH WAS NOT IMMUNIZED.

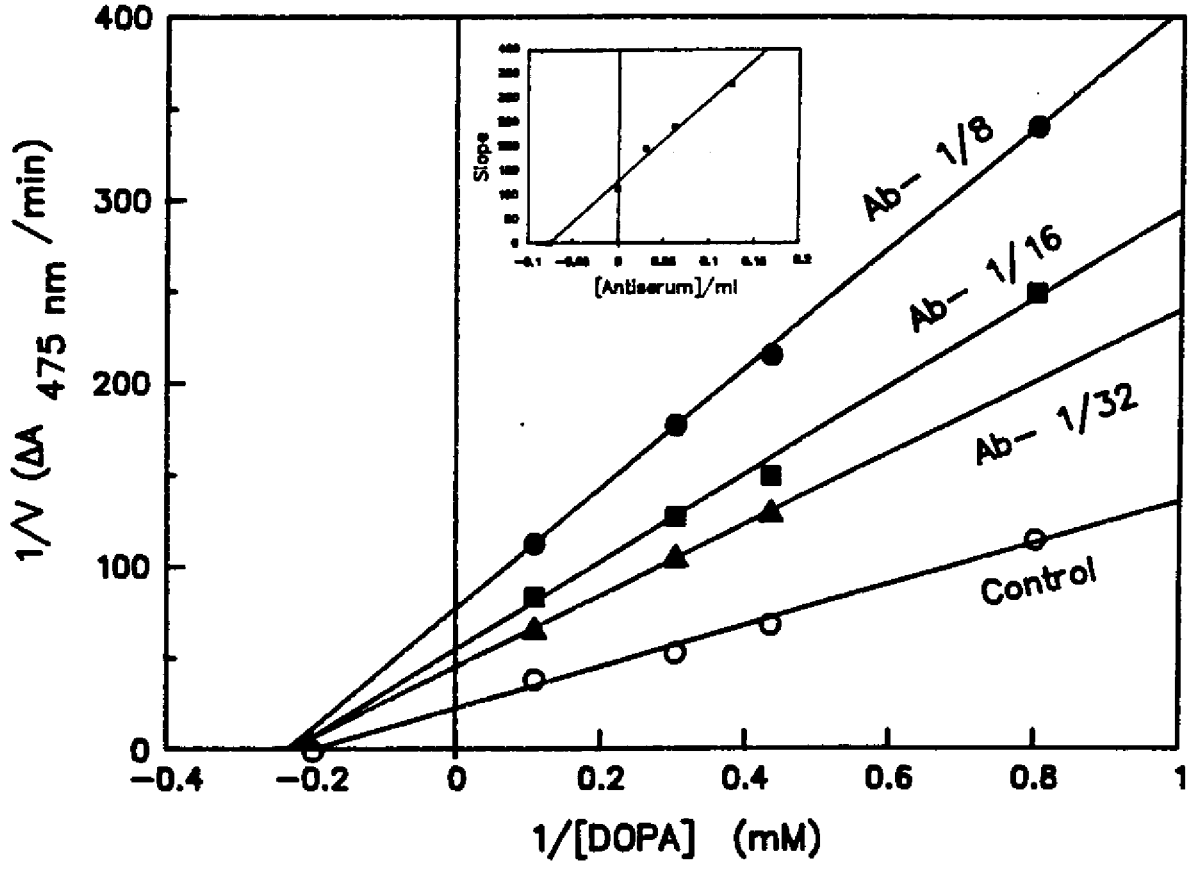

Figure 5. Double reciprocal plot of residual TAPO activity vs. DOPA concentration for various EAPO antiserum (S) concentrations pre−incubated with 0.026 units of TAPO activity. Assay conditions were as described in the methods.
Inset: Secondary plot of slope vs. antiserum concentration.

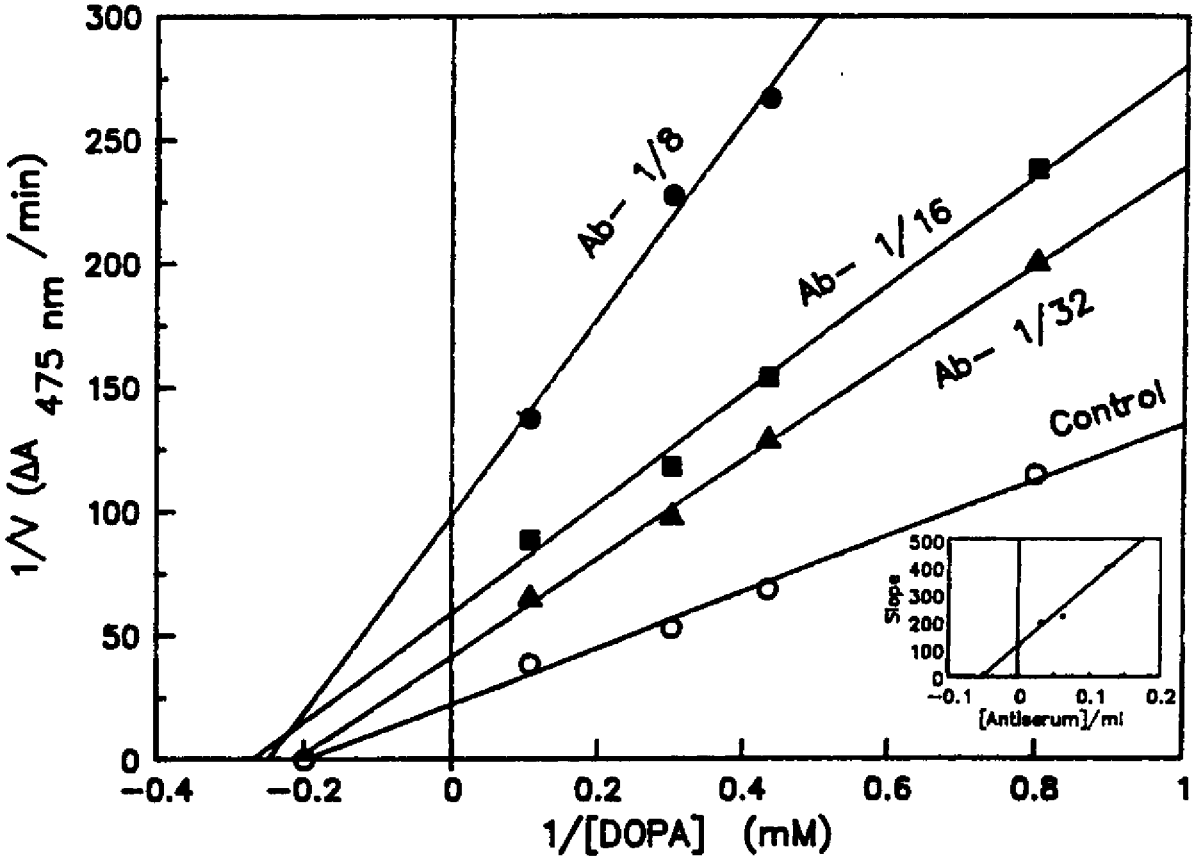

Figure 6. Double reciprocal plot of residual EAPO activity vs. DOPA concentration for various TAPO antiserum (T) concentrations pre-incubated with 0.026 units of EAPO activity. Assay conditions were as described in the methods.
Inset: Secondary plot of slope vs. antiserum concentration.

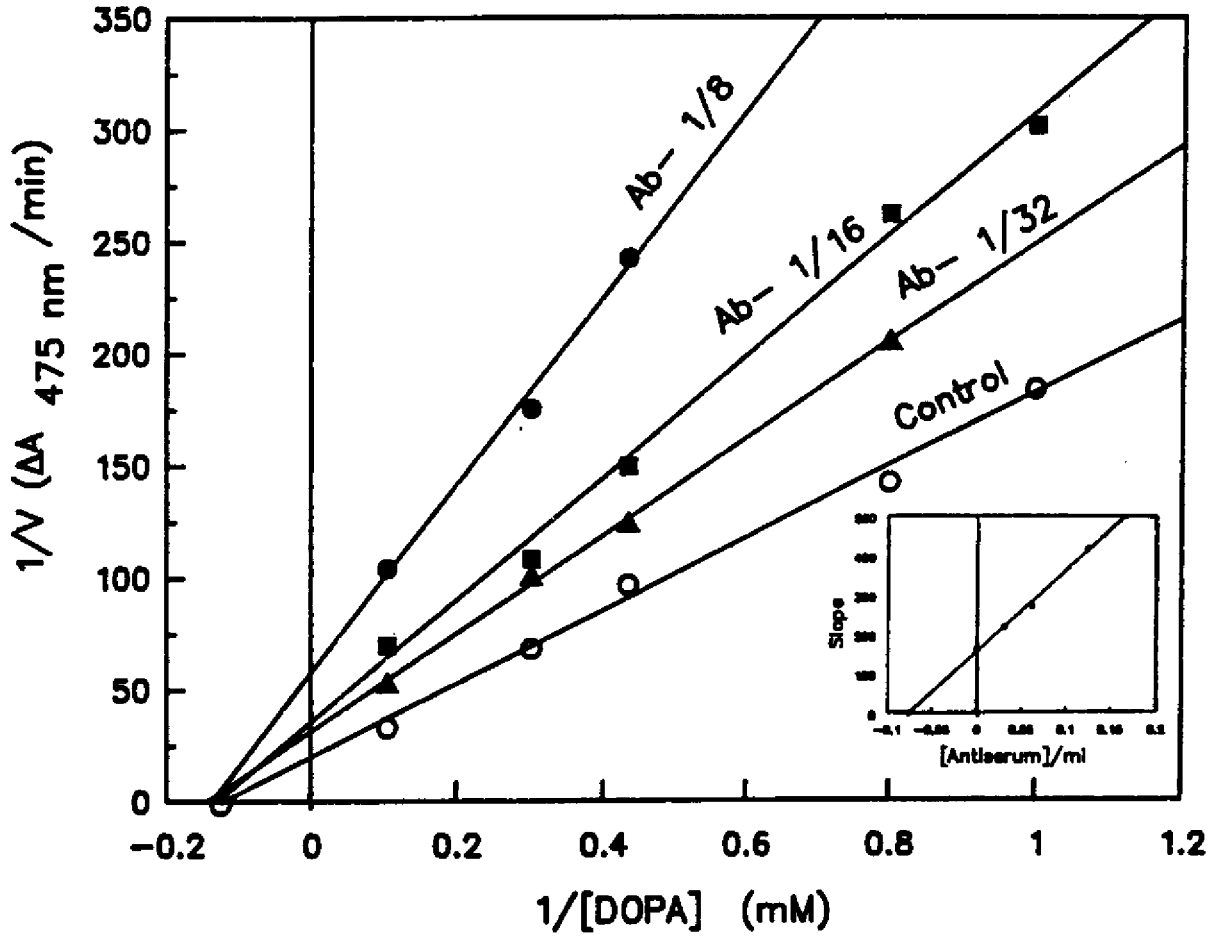

Figure 7. Double reciprocal plot of residual EAPO activity vs. DOPA concentration for various EAPO antiserum (S) concentrations pre-incubated with 0.029 units of EAPO activity. Assay conditions were as described in the methods.
Inset: Secondary plot of slope vs. antiserum concentration.

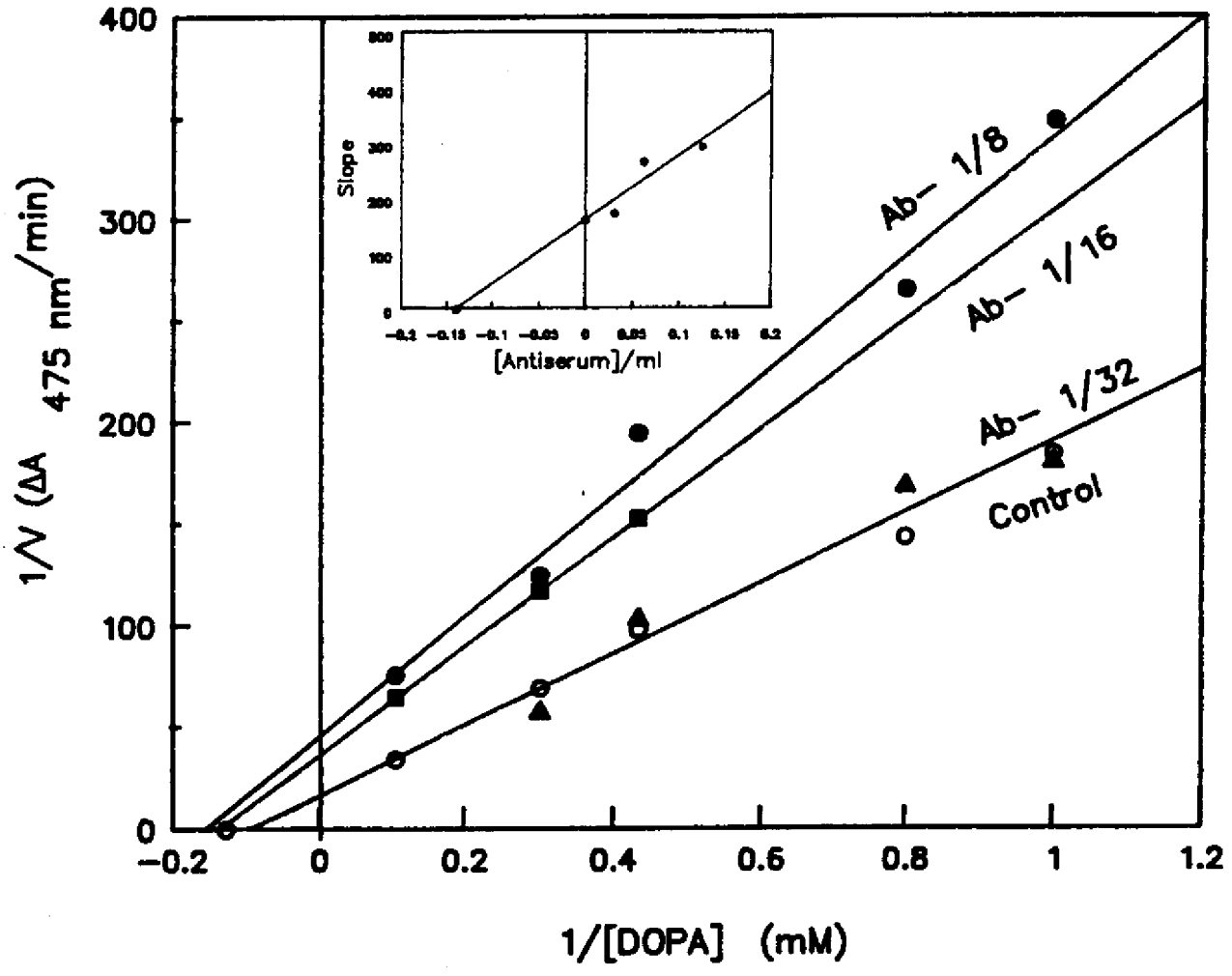

Figure 8. Double reciprocal plot of residual EAPO activity vs. DOPA concentration for various EAPO antiserum (S) concentrations pre-incubated with 0.029 units of EAPO activity. Assay conditions were as described in the methods.
Inset: Secondary plot of slope vs. antiserum concentration.

and the active sites of both enzymes were at differing locations within the enzyme structure.

Secondary plots of slope vs. antiserum concentration for the double-reciprocal plots were linear (Figs. 5-8), and formed the basis for computation of the relative dissociation constant (K_i) for the enzyme-antisera interaction (Table 1), expressed on the basis of the relative volume of standardized antiserum in one mL of potassium phosphate buffer pH 6.5. When pre-incubated with their respective antisera, a relative apparent K_i of 0.076 [antiserum/mL] was computed for both S and T antisera (Table 1), suggesting that enzyme-antiserum binding occured to a similar extent for both enzymes. However when allowed to cross-react, the S antisera pre-incubated with TAPO apparently formed a comparably more stable enzyme-antiserum complex (K_i = 0.049 [antiserum/mL]), while the T antisera pre-incubated with EAPO formed a relatively weaker enzyme-antiserum complex (K_i = 0.139 [antiserum/mL])(Table 1).

Table 1. Apparent K_i values obtained for EAPO and TAPO pre-incubated at 4°C with S and T antisera and subsequently assayed at 25°C.

TREATMENT	RELATIVE APPARENT K_i [antiserum/mL]*
S + EAPO	0.076
T + TAPO	0.049
T + EAPO	0.139
T + TAPO	0.076

Enzyme assay procedure was as described in the methods.

*[antiserum /m]) is equivalent to the volume of standardized antiserum preparation per mL of phosphate buffer pH 6.5

These differences in K_i for cross-reactivity point to possible structural differences between the EAPO and TAPO enzymes. Though both enzymes share identical antigenic sites, the locality of these antigenic sites within the protein structure may very well affect enzyme-antiserum binding, particularly when cross-reactivity is allowed to occur. These findings seem to re-enforce the theories of King and Flurkey (1987) that conformational differences in the protein structure are brought about by trypsin treatment. Further work will involve exploring the enzyme structure utilizing circular dichroism spectrophotometry.

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PURIFICATION AND CHARACTERIZATION OF A PROTEASE WITH TRYPTIC ACTIVITY FROM THE PYLORIC CECA OF MULLET (*MUGIL CEPHALUS*)

N.Guizani, R.S. Rolle, M.R. Marshall and C.I. Wei

Food Science and Human Nutrition Department
University of Florida, Gainesville, Fl 32611

INTRODUCTION

Trypsin is a proteolytic enzyme found in the digestive tract of many organisms. Over the past few decades, trypsin and trypsin-like enzymes have been identified and characterized in a wide array of vertebrates and invertebrates which include a number of stomached and stomachless fish (Overnall, 1973; Murakami and Noda,1981; Hjelmeland and Raa, 1982; Jany, 1976; Simpson and Haard, 1984 and 1987.) The recovery and purification of trypsin is of industrial interest. Approximately 50% of the enzymes used as industrial processing aids are protein hydrolases. Proteolytic enzymes can be used in a variety of ways to improve the quality, stability and solubility of foods as in baking, brewing, cheesemaking and meat processing industries.

Certain fish species lack a morphologically or physiologically distinct stomach, and are thus deprived of the acid denaturation and acid hydrolysis which takes place in the stomach, thus making protein in the feed more amenable to subsequent degradation in the intestine by trypsin, chymotrypsin and other proteases or peptidases. According to Beauvalet (1933) intestinal secretions from stomached fish are not adequate in themselves to digest protein, whereas intestinal secretion from stomachless fish are sufficient to facilitate complete protein digestion. This observation is consistent with the recent view that such fish secrete enzymes which are more efficient in catalyzing hydrolysis of native proteins.

The present paper describes the purification and properties of a protease from the pyloric ceca of mullet, a stomachless fish.

MATERIALS AND METHODS

Extraction of trypsin:

Mullet (*Mugil cephalus)* was obtained from a local retail seafood store.

The procedure used to extract the enzyme was that of Simpson and Haard (1984) with slight modification. Mullet pyloric ceca were frozen in liquid nitrogen, powdered in a Waring blender, and mixed with

extraction buffer (0.05M Tris HCl containing 0.5M NaCl and 0.02M $CaCl_2$ pH 7.8) in a ratio of 1:5 (w/v). The slurry was stirred for 4 hours at 4^0C and centrifuged at 3000*g* for 30 min at 4^0C. Brij 35 was added to the supernatant to a final concentration of 0.02%. The supernatant was stirred overnight at 4^0C and finally centrifuged at 10,000*g* for 30 min at 4^0C. The sediment was discarded, the supernatant collected was fractionated with solid ammonium sulfate at 4^0C and the precipitate forming between 20 and 40% of saturation was dissolved in a minimum amount of extraction buffer and dialysed against the extraction buffer overnight at 4^0C. Three volumes of cold acetone (-20^0C) were added and the precipitate formed was collected by centrifugation at 6000*g* for 30 min at 4^0C. The acetone precipitate was dried first with cold (-20^0C) acetone-ether (1:1 v/v) and then cold (-20^0C) peroxide-free ether. The dried material was suspended in a minimum amount of extraction buffer and centrifuged at 10,000*g* for 30 min at 4^0C. The supernatant obtained was incubated at 4^0C for 24 hours prior to affinity chromatography.

Affinity chromatography:

The acetone fraction was pumped onto a column packed with 10 mL of p-aminobenzamidine-sepharose at a rate of 15 mL/h and subsequently eluted with extraction buffer until no additional protein was detected in the eluent. The trypsin fraction was desorbed from the column by washing with 5 mM HCl. The fraction retained by the p-aminobenzamidine was pooled, dialyzed against the extraction buffer, concentrated using an amicon cell (10 KDa cut off; Pharmacia) and pumped onto a column packed with 10 mL soybean trypsin inhibitor SBTI-sepharose. Elution conditions were as described for p-aminobenzamidine-sepharose. The protein fraction eluted from the SBTI-sepharose support with 5 mM HCl was designated "trypsin".

Basic assay for trypsin activity:

Amidase activity was determined according to the method of Erlanger et al. (1961) using N-α-Benzoylarginine-p-nitroanilide (BAPA) as substrate. One BAPA unit of activity was defined as $\Delta A_{410nm/min}$ x 1000 x 3 divided by 8800-where 8800 is the extinction coefficient of p-nitroaniline (Erlanger et al., 1961). Esterase activity was determined spectrophotometrically using tosylarginine methyl ester (TAME) as substrate in accordance with the method of Hummel (1959). One TAME unit of activity was defined as $\Delta A_{247nm/min}$ x 1000 x 3 divided by 540-where 540 is the extinction coefficient of tosyl arginine (Anonymous, 1978).

Protein determination:

Protein determinations were based on the dye-binding assay as described in the Bio-Rad protein standard assay bulletin (Bio-Rad innstruction Manual 82-0275-1282, Bio-Rad laboratories, Richmond, CA).

Electropheresis:

SDS-PAGE of the trypsin was performed according to the method of Laemmli (1970).

Trypsin inhibition:

Inhibition of trypsin by phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), aprotinin and benzamidine was investigated according to the method of Simpson and Haard (1984). Only one level of SBTI (0.1 mg/mL) and one aprotinin concentration (0.5 trypsin inhibitor units/mL) was tested.

pH optima and stability:

The pH optimum of the trypsin was determined by preparing the substrate (BAPA) in various buffer solutions and applying the extracts to the substrates individually. Residual amidase activity was assayed by measuring the initial rate of release of p-nitraniline at 410 nm and a temperature of 25°C. pH stability of the trypsin was determined by the pre-incubating it in various buffer solutions in an ice bath for 30 min. Residual amidase activity was assayed at 25°C with BAPA (pH 8.2) as a substrate. The composition of the buffer solutions used were: 0.1 M citrate-HCl, pH 2.0; 0.1 M citrate-NaOH, pH 4.0; 0.1 M citrate-NaOH, pH 6.0; 0.1 M Tris-HCl, pH 7.0; 0.1 M Tris-HCl, pH 7.5; 0.1 M Tris-HCl, pH 8.0; 0.1 M Tris-HCl, pH 8.5; 0.1 M Tris-HCl, pH 9.0; 0.1 M glycine-NaOH, pH 10.0.

Temperature optimum and thermostability:

The temperature optimum of the trypsin was determined by measuring amidase activity at various temperatures. The initial slope of the reaction was used to calculate BAPA activity.

In order to determine thermostability, the trypsin was pre-incubated at various temperatures for 30 min, rapidly cooled in an ice bath for 5 min, and assayed for residual amidase activity at 25°C.

RESULTS AND DISCUSSION

Purification of the trypsin from mullet pyloric ceca is summarized in Table 1. The specific activity of the purified trypsin was about 90 times greater than that of the crude extract corresponding to a yield of 16%, on the basis of its BAPA activity.

Polyacrylamide gel electrophoresis in the presence of SDS resolved the trypsin into a single distinct protein band. The molecular weight of the trypsin was estimated to be approximately 24,000, which is in accordance with that for trypsin enzymes. According to Keil (1971) trypsin enzymes have a molecular weight ranging from 20,000 to 24,000 DA.

Table 1: Purification scheme of mullet trypsin from Pyloric ceca.

Step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield %	Purification
'Sup' 1	1295.00	19.60	0.0148	100.0	1.0
'Sup' 2	941.00	19.47	0.0207	101.0	1.4
Ammonium sulfate fraction	88.11	18.27	0.2100	93.2	14.0
Acetone fraction	26.93	11.41	0.4237	59.6	29.0
Aminoben -zamidine fraction	3.77	4.26	1.1300	22.3	76.0
SBTI fraction	2.32	3.15	1.3560	16.4	92.0

The optimum pH for hydrolysis of BAPA at 25^{0}C by the trypsin was 8.0 (Fig 1). This value was similar to those previously reported for invertebrate trypsins (Jany,1976; Hjelmeland and Raa, 1982; Gates and Travis,1969; Simpson and Haard,1984). Mullet trypsin exhibited optimum stability over a pH range of 7.5-9.0 (Fig 2.). Most trypsins from fish and other lower vertebrates or invertebrates, are in fact very unstable under acidic conditions but very stable at neutral to slightly alkaline conditions (Jany,1976; Simpson and Haard,1984; Hjelmeland and Raa,1982).

Mullet trypsin exhibited a temperature optimum for amidase activity at 55^{0}C (Fig 3.). This temperature is slightly higher than those reported for trypsins from certain fish (Simpson and Haard,1984; Hjelmeland and Raa,1982) but lower than those reported for trypsins from mammals (Erlanger et al,1961: Simpson and Haard, 1984). The effect of temperature on the stability of the enzyme is shown in Fig. 4. The enzyme was relatively unstable to temperature and lost about 40% of its activity after heating for 30 min at 65^{0}C, and all of its activity at 75^{0}C. These results are similar to those reported for trypsins from marine organisms which are thermolabile as compared to bovine trypsin which has been reported to be resistant to thermal degradation.

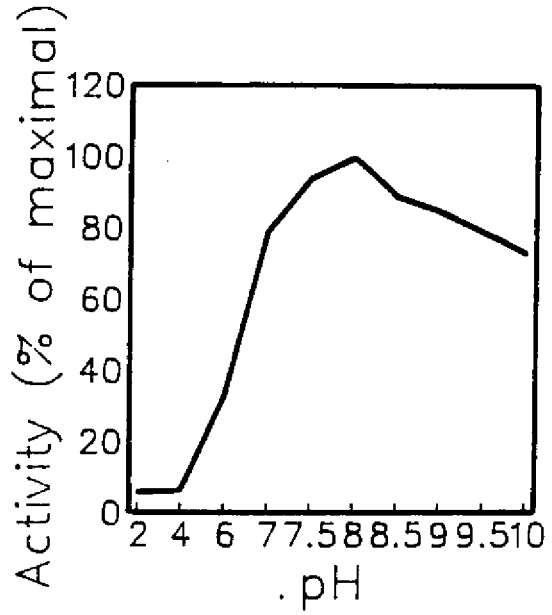

Fig 1: pH optimum of Mullet trypsin with BAPA at 25 C.

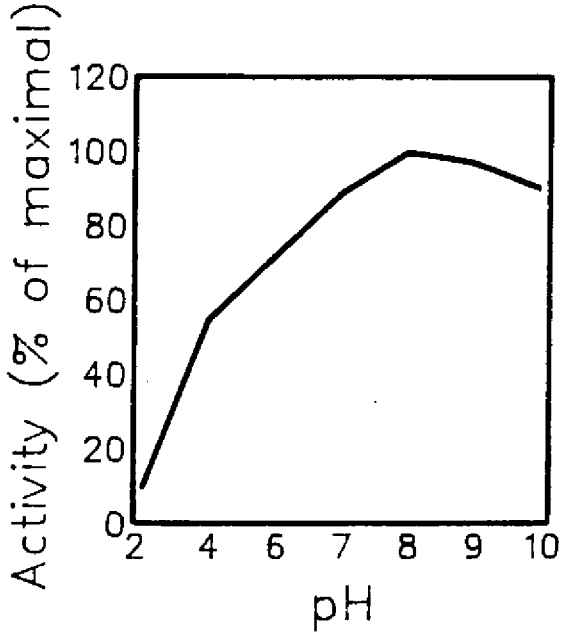

Fig 2: pH stability of Mullet trypsin on BAPA at 25 C.

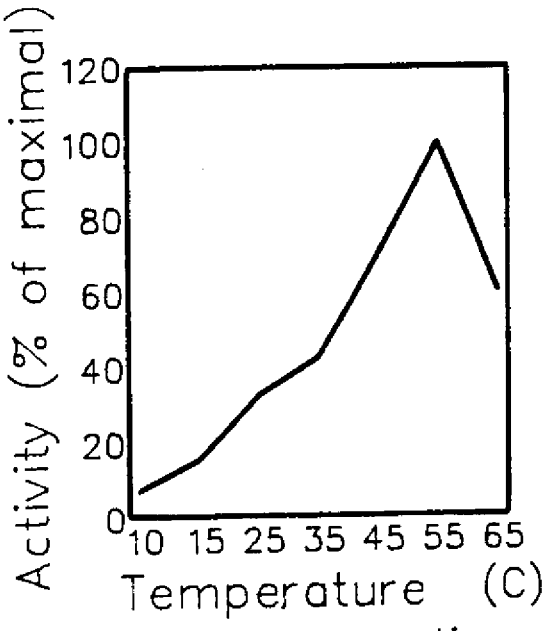

Fig 3: Temperature optimum of Mullet trypsin on BAPA

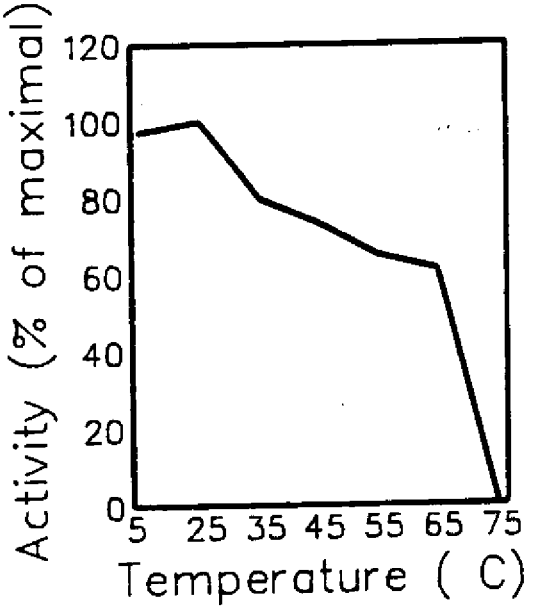

Fig 4: Thermostability of Mullet trypsin using BAPA as substrate.

The influence of various serine protease inhibitors is summarized in Table 2. The protease obtained from mullet was dramatically inactivated by PMSF, SBTI, aprotinin and benzamidine. Inhibition of the enzyme by PMSF suggests that it is a serine protease like other trypsins and trypsin-like enzymes (Jany, 1976; Hjelmeland and Raa,1982; Simpson and Haard,1987). Inhibition of the enzyme by SBTI and aprotinin suggests that the enzyme had a similar mechanism of substrate binding by its active centers to other trypsins (Gates and Travis,1969; Simpson and Haard,1987), while inhibition of the enzyme by benzamidine suggests that its active center is homologous to that of authentic trypsins since the center of authentic trypsins is known to bind with guanidines and amidines (Mihalyi,1978; Mares-Guia and Shaw,1965).

Table 2: Inhibition of mullet trypsin by known trypsin inhibitors.

Inhibitor	Substrate	Concentration	% Inhibition
PMSF	TAME	5.00 mM	62
SBTI	BAPA	25.00 mM	93
Aprotinin	TAME	0.25 (TIU/mL)	96
Benzamidine	BAPA	2.50 mM	73

CONCLUSION

On the basis of the results obtained , the enzyme isolated is classified as a trypsin. Further studies in our laboratories will be designed to determine the possible use of mullet trypsin as a food processing aid.

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ISOLATION AND CHARACTERIZATION OF TWO TRYPSIN-LIKE ENZYMES FROM MENHADEN, Brevoortia tyranus, INTESTINE

J.H. Pyeun*, H.R. Kim and J.S. Godber

Department of Food Science
Louisiana Agricultural Experiment Station
LSU Agricultural Center
Baton Rouge, LA 70803

* Current address: Department of Food Science & Nutrition
National Fisheries University of Pusan
Pusan, Korea

INTRODUCTION

Menhaden, Brevoortia tyranus, which contributed over 50% of all finfish harvested in the United States in 1987 is one of the major resources for fish oil production and the animal feed industry. High activity of proteases in fish intestines accelerates autolytic degradation of abdominal tissues, which causes the fish to become unsuitable as a raw material for conventional fish food processing and sometimes even for meal and oil production (Gildberg, 1978; Gildberg and Raa, 1979). Trypsin is quantitatively very important in the digestive system. Furthermore, the participation of other proteases in muscle degradation is affected by the presence of trypsin.

A survey of proteolytic digestive enzymes in various species of fish has revealed that a serine protease is widely distributed in fish intestine. Trypsin from fish intestine was isolated and characterized in anchovy (Martinez et al., 1988), mackerel (Kim and Pyeun, 1986), capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka et al., 1983), sardine (Murakami and Noda, 1981), and various crustacea (Kim et al., 1988; Kimoto et al., 1983; Zwilling and Neurath, 1981; Lee et al., 1980). Among the proteases found , trypsin-like enzyme was generally characterized with regard to physicochemical, inhibitory and kinetic properties (Simpson and Haard, 1984; Cohen, et al., 1981; Reeck and Neurath, 1972).

In a preliminary study, it was noted that menhaden abdominal tissues were severely degraded at refrigerated temperature and the fish had high activity of trypsin-like enzyme. In order to understood the effect of intestinal trypsin on muscle degradation in menhaden, it was necessary to establish isolation procedures

and determine biochemical properties of menhaden trypsin-like enzymes.

MATERIALS & METHODS

MATERIAL

Fresh menhaden, Brevoortia tyranus, was harvested in the Gulf of Mexico. The intestine of fresh menhaden was separated on board the fishing vessel and transported to the laboratory under dry ice.

Extraction of Proteinase

One hundred grams of viscera were homogenized in 400 ml of 1% NaCl containing 1 mM disodium ethylenediaminetetraacetate with an Ultra-Turrax type tissue grinder (Junke and Kunkel Co.) for 30 s. The extract was held at 37°C for 3 hr and centrifuged at 10,000 x g for 30 min. The supernatant was dialyzed overnight against distilled water and centrifuged. The crude enzyme solution was obtained after centrifugation.

Isolation of Trypsins from Menhaden Intestine

During the purification steps, tryptic activity was determined by amidase activity using benzoyl-arginine-p-nitroanilide (BAPNA). Purification of trypsins from crude enzyme solution was undertaken by first employing ammonium sulfate fractionation with 30-70% saturation. The suspension was dialyzed against 50 mM Tris-HCl buffer, pH 6.8, containing 0.5 M NaCl, 5 mM $CaCl_2$ and 1 mM benzamidine. The dialysate was applied to a benzamidine-Sepharose 6B column (1.5 x 10 cm) previously equilibrated with the above buffer but not containing benzamidine. The column was eluted with the equilibration buffer until the effluent did not contain protein. Trypsin-like enzyme was then eluted from the column using 50 mM Tris-HCl, pH 6.8, containing 0.5 M NaCl, 5 mM $CaCl_2$ and 125 mM of benzamidine. The protease fractions were pooled and dialyzed against 0.01 M Tris-HCl buffer, pH 6.8, and applied to a DEAE-Sephacel column (3 x 50 cm) equilibrated with the same buffer solution. The column was eluted with a 2000 ml linear gradient ranging from 0 to 0.4 M NaCl. Two protease fractions were separated by this chromatography and each fraction was concentrated and dialyzed against 10 mM Tris-HCl, pH 6.8 containing 0.1 M NaCl. The dialyzed solution was applied to a Sephadex G-75 column (2.5 x 90 cm) and eluted with the same buffer. The fraction (Sephadex G-75) with high tryptic activity was concentrated with ultrafiltration and stored at -20°C until use in subsequent characterization studies.

Determination of Amidase Activity

Amidase activity was measured from the hydrolysis of benzoyl D,L-arginine p-nitroanilide (BAPNA) in an assay method described by Erlanger et al. (1961). The

reaction mixture consisted of 25 μl of enzyme and 0.5 ml of 1 mM BAPNA in 0.05 M Tris-HCl buffer, pH 8.1, containing 0.01 M $CaCl_2$. One enzyme unit was defined as the amount of enzyme that hydrolyzed 1 μmole of BAPNA per min under the conditions described above. Specific activity was expressed as enzyme units per mg of protein.

Protein Concentration

Protein concentration was determined by the method of Lowery et al., (1951) using bovine serum albumin as standard.

Electrophoresis

Disc-polyacrylamide gel electrophoresis at pH 8.3 was carried out according to the method of Davis (1964).

Estimation of Molecular Weight

Molecular weights of two purified trypsins were determined by SDS-PAGE according to the modified method of Laemmli (1974) using phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrolase as standards.

pH and Temperature Optima

The effect of pH was measured at an enzyme concentration of 20 μg/ml using 1 mM BAPNA at pH values in the range of pH 6.0 to 12.0. Temperature dependence was examined at pH 8.1 in the temperature range of 20-70^{o}C.

Effect of Inhibitors

Tosyl-L-lysine chloromethyl ketone (TLCK, 2.5 μM), benzamidine (1.25 mM), diisofluorophosphate (DFP, 1 mM), soybean trypsin inhibitor (SBTI, 0.15 mg/ml), leupeptin (2.15 μg/ml), antipain (8.5 μg/ml), phenylmethylsulfonyl fluoride (PMSF, 5.0 mM), tosyl-L-phenylalanine chloromethyl ketone (TPCK, 1.25 mM) and ethylenediaminetetraacetate (EDTA, 0.5 mM) were added separately to an equal volume of the trypsin solutions (30 μg/ml) and incubated at 30^{o}C for 15 min. After incubation, residual tryptic activity was determined with BAPNA.

RESULTS & DISCUSSIONS

Purification of Trypsins from Menhaden Intestine

A chromatogram of a DEAE-Sephacel chromatography of the proteolytic fraction obtained from benzamidine Sepharose 6B column is shown in Fig. 1. With this ion-exchange chromatography, two fractions designated proteinases A and B were eluted separately at the ionic strength of 0.14 and 0.35, respectively. The pooled and concentrated fraction of each was purified using gel filtration with Sephadex G-75 (Fig. 2). The specific activity and recovery of proteinase A and B during purification is illustrated in Table 1. The results obtained from 100 g of

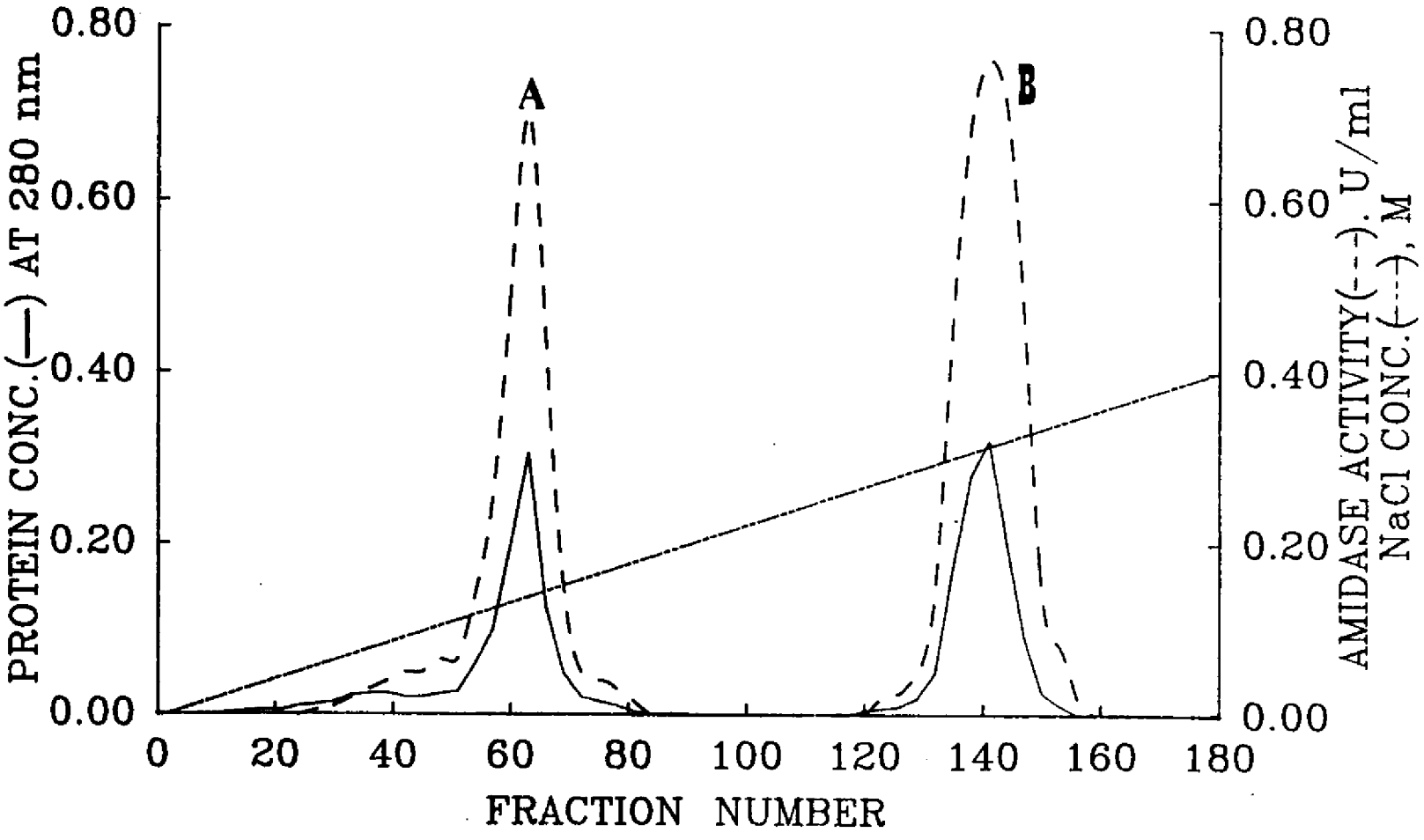

Fig. 1. Chromatogram of DEAE-Sephacel fractionation of the trypsin fraction obtained from benzamidine Sepharose-6B column. The flow rate was 40 ml/hr and fraction volume was 10 ml per tube.

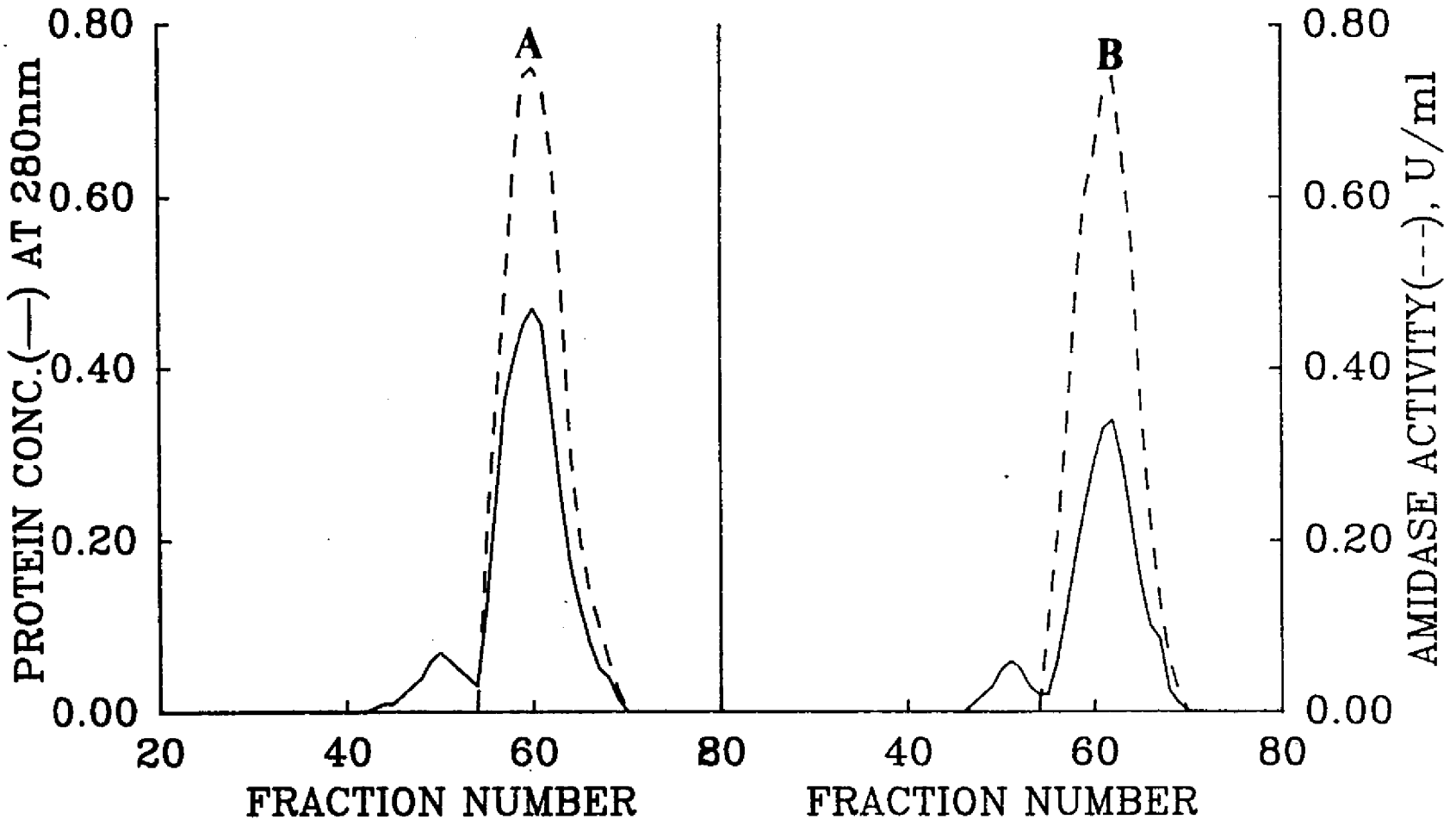

Fig. 2. Chromatogram of Sephadex G-75 fractionation of the trypsin fraction A and B obtained from DEAE-Sephacel chromatography. The flow rate was 15 ml/hr and fraction volume was 5 ml per tube.

menhaden intestine indicated that proteinase A was purified 15.7-fold with 23% recovery and proteinase B was purified 6.8-fold with 16% recovery. The combined recovery was 39%. The purification procedure was more effective for the isolation of trypsin-like enzyme from fish intestine than the method for mackerel (Pyeun and Kim, 1986), skipjack (Pyeun et al., 1987) and anchovy (Martinez et al., 1988). Disc-polyacrylamide electrophoresis of the purified enzymes showed a single band for each enzyme fraction which is evidence for the homogeneity of two proteinases. (Fig. 3).

Table 1. Purification of proteinase A and B from menhaden intestine

Fraction	Volume (ml)	Protein (mg/ml)	Specific Act.(U/mg)	Yield %	Purity
Crude enzyme	474	1.56	0.28	100	1.0
A.S. Fraction	86	2.98	0.66	80	2.4
Benzamidine Sepharose 6B	28	2.04	2.67	72	9.5
DEAE-Sephacel					
Enzyme A	16	0.94	3.47	24	12.4
Enzyme B	12	1.75	1.77	17	6.3
Sephadex G-75					
Enzyme A	7	1.57	4.40	23	15.7
Enzyme B	8	2.25	1.91	16	6.8

A.S.-Ammonium sulfate

Determination of Molecular Weight

The molecular weights of the two proteinases were estimated by SDS-polyacrylamide electrophoresis (Fig.4) to be 26,200 D and 25,000 D, respectively. This result was similar to that of bovine trypsin and those of other fish trypsins (Cohen et al., 1981; Reeck and Neurath, 1972). Also, molecular weights around 27,000-30,000 D have been reported for trypsins from sardine (Murakami and Noda, 1981) and capelin (Hjelmeland and Raa, 1982).

Effect of pH and Temperature on Amidase Activity

The maximum activity was found in the pH range of 8.0 to 10.0 for both enzymes (Fig.5). Also, maximum amidase activity was obtained at 45°C. Proteolytic activity from the intestine of anchovy (Heu, 1988), skipjack (Pyeun et

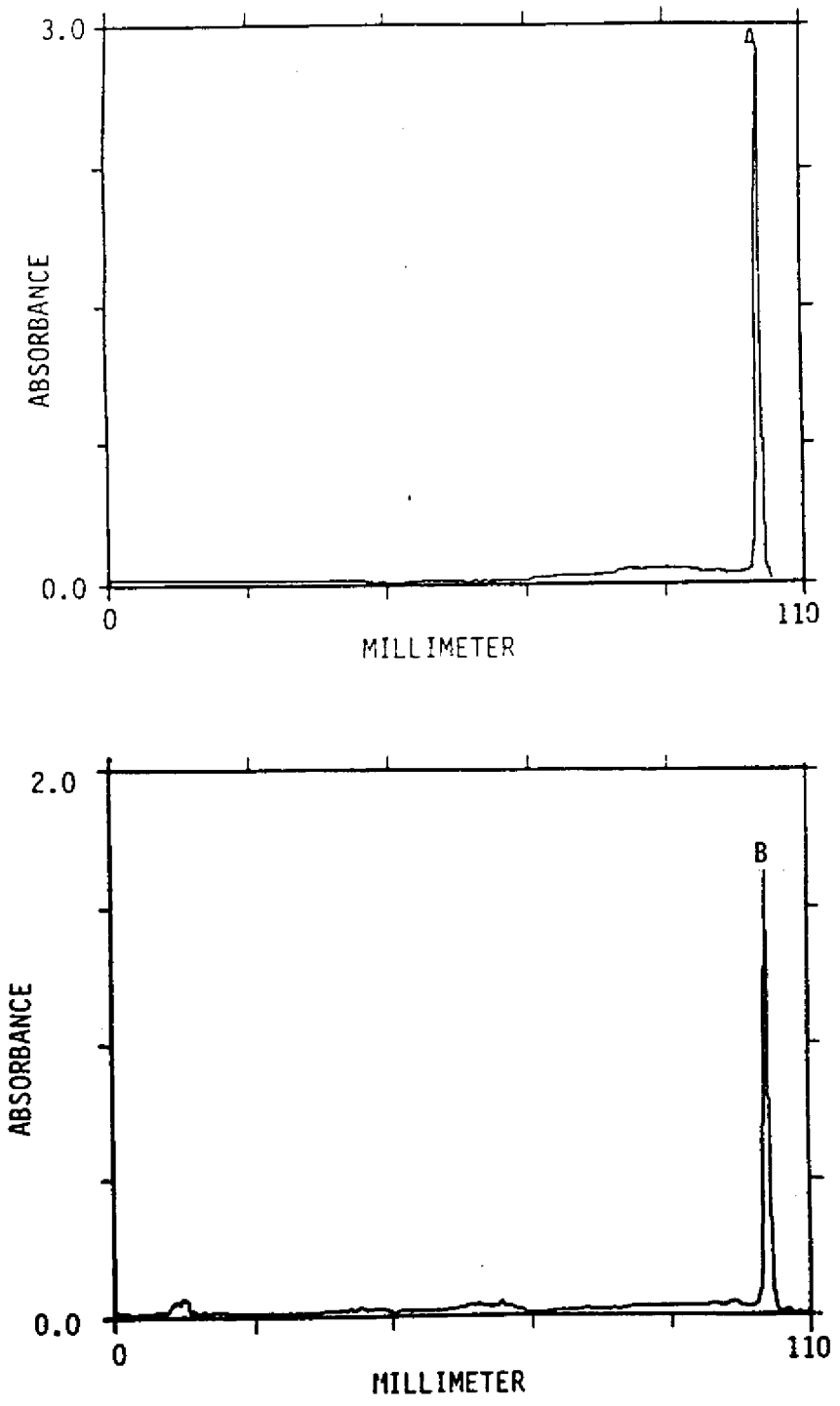

Fig. 3. Densitograms of disc-gel electrophoretic pattern of trypsin A and B from menhaden intestine. Scanning was performed at 600 nm.Electrophoresis was performed with 7.5% polyacrylamide gel (0.5 x 12 cm) at pH 8.3 with 4 mA per column for 4 hrs.

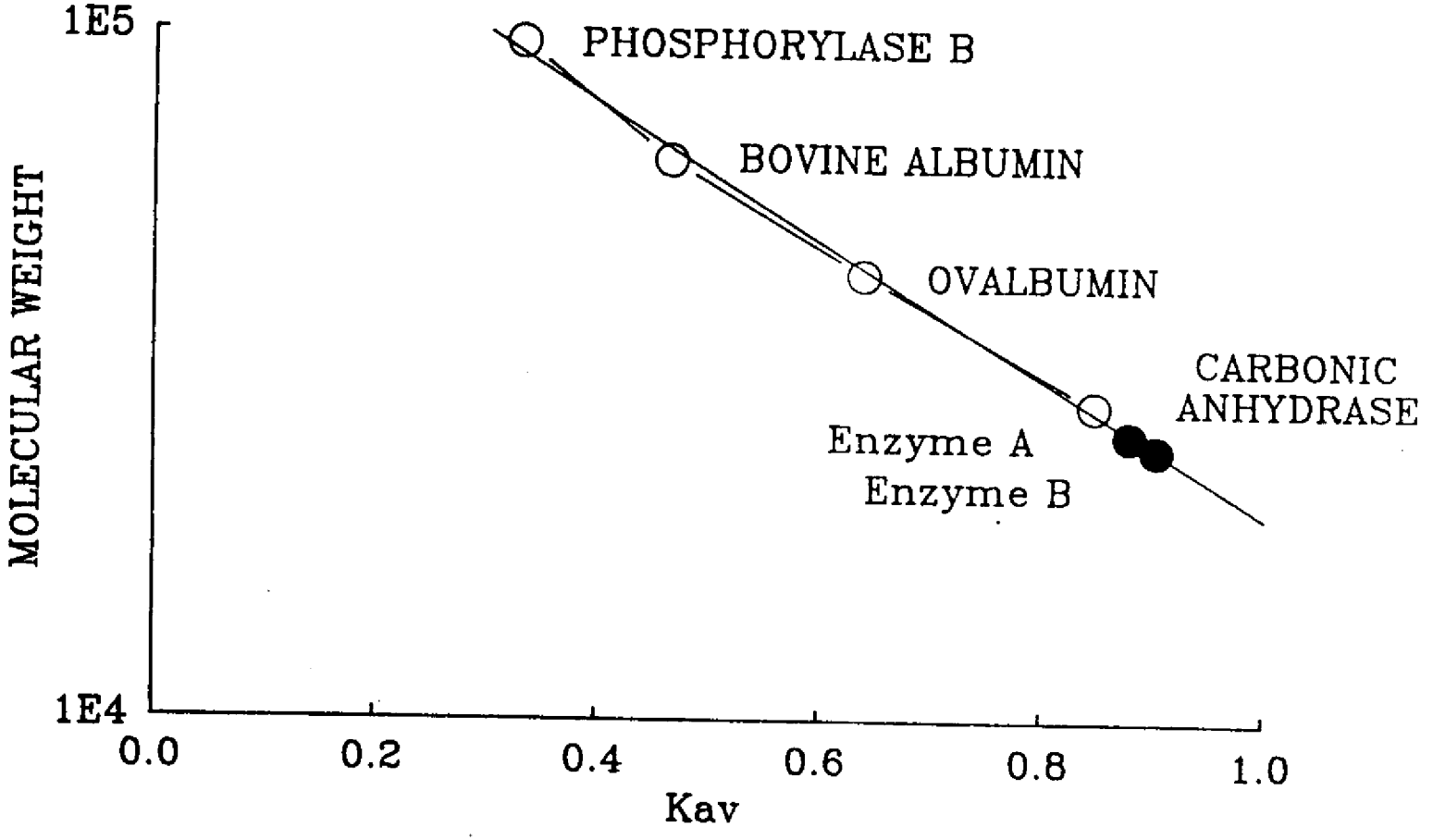

Fig. 4. Estimation of molecular weights of the menhaden trypsins by SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed with 7.5% polyacrylamide gel containing 0.1% SDS. Standard proteins used were phosphorylase B, bovine serum albumin, ovalbumin and carbonic anhydrolase.

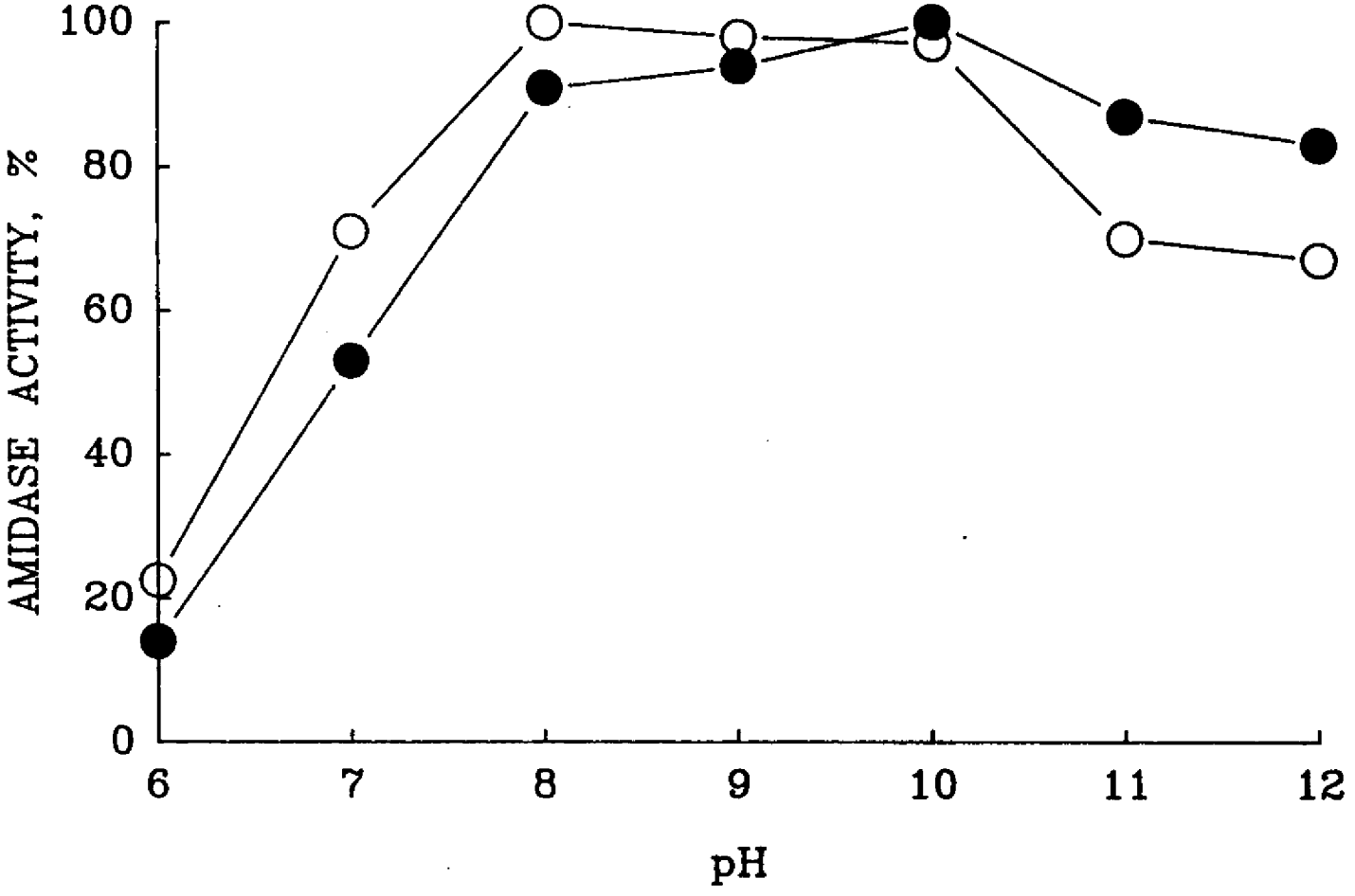

Fig. 5. Effect of pH on the hydrolysis of BAPNA by enzyme A and B from menhaden intestine. The buffers used were 0.1 M phosphate (pH 6.0-7.0), 0.1 M Tris-HCl (pH 7.0-8.5) and 0.1 M sodium carbonate (pH 8.5-10.5) and glycine-NaOH buffer (pH 10.5-12.0).

al., 1988), mackerel (Kim and Pyeun, 1986), and sardine (Murakami and Noda, 1981) showed optimum activity at a similar pH and temperature. At the optimum reaction condition, the specific activity of proteinase A and B was 1.67 U/mg protein and 1.09 U/mg protein, respectively, which were lower than three other alkaline proteinases reported from mackerel pyloric caeca (Kim and Pyeun, 1986).

Effect of Inhibitors on Enzyme Activity

Both proteinases were inhibited completely by tosyl-lysine chloromethyl ketone (TLCK), diisofluorophosphate (DFP) and benzamidine (Table 2), which are specific inhibitor of trypsin. Neither were inhibited by tosyl-phenylalanine chloromethyl ketone, which is a specific inhibitor of chymotrypsin. Also, both were inhibited by soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), leupeptin and antipain, which are inhibitor of serine protease. These results suggest that proteinase A and B are trypsin-like enzymes. Furthermore, 2Na-EDTA inhibited the amidase activity of both enzymes suggesting that further study is needed to clarify the activation of the enzymes by Ca^{++} ion.

Thus, we have established a purification procedure having high recovery of trypsin from menhaden intestine. Two trypsins with enzymatic and physicochemical properties similar to other trypsin from fish intestine were characterized.

Table 2. Effect of inhibitors on the amidase activity of trypsin-like enzyme A and B from menhaden intestine

Inhibitor	Conc.	Relative activity, %	
		Enzyme A	Enzyme B
None		100	100
Tosyl-L-lysine chloromethyl ketone	2.5 mM	0	0
Tosyl-L-phenylalanine chloromethyl ketone	1.25 mM	100	100
Soybean trypsin inhibitor	0.15 mg/ml	0	0
Diisofluorophosphate	1.0 mM	0	0
Benzamidine	1.25 mM	14	43
Phenylmethylsulfonyl fluoride	2.0 mM	84	56
Ethylenediaminetetraacetate	0.5 mM	14	0
Leupeptin	2.15 μg/ml	0	0
Antipain	8.50 μg/ml	0	0

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DEVELOPMENT OF ROCK SHRIMP SPECIFIC MONOCLONAL ANTIBODY FOR ROCK SHRIMP IDENTIFICATION

C.I. Wei[1], H. An[1], M.R. Marshall[1], W. S. Otwell[1], K.J. Kao[2], and P.A. Klein[2]

Food Science and Human Nutrition Department[1] and Department of Pathology and Laboratory Medicine[2], University of Florida, Gainesville, FL 32611

INTRODUCTION

There has been a tremendous growth in seafood consumption in this country due to changes in consumer attitudes toward health and nutrition. With the increased demands for higher quality seafood products, consumers may have already encountered the willful or unintentional adulteration by dealers who substitute higher quality and priced seafood with lower quality and less expensive products.

Identification of seafood species can be difficult. The use of morphological characteristics for identification requires a great deal of experience; unintentional fraud can occur when identification is done by untrained personnel, and the resulting products may be declared misbranded and/or adulterated by regulatory agencies (Vondruska, 1988). Several electrophoretic methods are used officially to differentiate seafood species or seafood products (AOAC, 1984). However, these methods are laborious, time-consuming and require substantial equipment. They are therefore limited for field test applications.

Immunological assays, as an alternative, can be used to reduce the test time and cost, as well as to increase the sensitivity in detecting food components (Hayden, 1977, 1978, and 1981; Hitchcock et al., 1981; Karpas et al., 1970; Skerritt, 1985). Lundstrom (1984, 1985), using hybridoma technology, developed the enzyme-linked immunoosorbent assay (ELISA) to identify fish species and to differentiate fish stock. Crude protein extracts from heat-denatured fish muscle were used to immunize mice for the preparation of monoclonal antibodies (McAbs). However, high cross-reactivity of the antibodies was noted between unrelated fish species, and species-specificity was not found when McAbs were tested against numerous fish species. This might have been due to the fact that most of the antibodies produced against the crude extracts were specific for most of the prevalent proteins, rather than the less abundant species-specific proteins.

ELISA has been widely used for detecting the species origin of fish, beef, pork, and chicken or to detect food components in seafood or meat products (Hayden, 1977, 1978 and 1981; Hitchcock et al., 1981; Karpas et al., 1970; Skerritt, 1985). ELISA offers great advantages in that (1) the test can be performed in a short period of time, (2) a small volume of species-specific antisera is required, (3) the antisera can be mixed for multi-specificity in a screening procedure, (4) equipment is available to semi-automate the assay, and (5) the increased sensitivity of the assay allows the use of simple sampling techniques (Whittaker et al., 1983).

The present study was carried out to develop rock shrimp (*Sicyonia brevirostris*) specific McAb using isolated rock shrimp proteins as antigen, and

to use this specific McAb as a model to increase the specificity of the ELISA for species identification. The applicability of the ELISA to identify rock shrimp among seafood and meat samples, and quantify rock shrimp content in various protein mixtures was also investigated.

MATERIALS AND METHODS

Preparation of water-soluble proteins

Water-soluble proteins were extracted from 23 different seafood and meat samples, including rock shrimp, as previously reported (An et al., 1988). Samples were chopped, and mixed at a ratio of 1:3 (w/v) with an aqueous solution containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% sodium azide. The samples were homogenized using a Polytron (setting 5.5) at room temperature for 1 min and then centrifuged at 48000 x *g* for 20 min at 20°C. The supernatants were collected, and the protein contents determined (Lowry et al., 1951).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to An et al. (1988) using the Bio-Rad Protean II unit. Proteins were stained with Coomassie Brilliant Blue R-250. Electro-phoretic patterns were recorded by developing the positive image using a Kodak Electrophoresis Duplicating Paper. A low molecular weight protein kit (Pharmacia) was used as protein standards, which contained phosphorylase b (94000 Da), bovine serum albumin (67000 Da), ovalbumin (43000 Da), carbonic anhydrase (30000 Da), soybean trypsin inhibitor (21100 Da) and α-lactalbumin (14400 Da).

Purification of protein C

Rock shrimp-specific proteins with MW of 17.7 and 18.5 kD (An et al., 1988), referred to as protein C, were eluted from the 12% SDS-PAGE slab gels of rock shrimp extract. The gels were homogenized in water for 1 min using a Polytron, and the mixtures centrifuged at 2000 x g for 20 min at room temperature. The eluted proteins were pooled and dialyzed overnight at 4°C in a membrane tubing (mol wt cutoff 6000-8000 Da) against the phosphate buffered saline (PBS, pH 7.4) containing 1.4 M NaCl, 0.1 M Na_2HPO_4 and 0.3 M KH_2PO_4. The dialyzed protein "C" was analyzed for protein content and purity using SDS-PAGE.

Two-dimensional gel electrophoresis of rock shrimp water extract

Two-dimensional electrophoresis using urea IEF in the first dimension and SDS-PAGE in the second dimension was performed as previously described (An et al., 1988, 1989). The first dimension using rock shrimp protein extract at 150 μg was done with a tube gel (11.5 cm x 1.7 mm) and a Pharmacia Electrophoresis Apparatus GE-4II unit. After the focused gel was removed and equilibrated in 0.125 M Tris-HCl buffer (pH 6.8) for 1 hr, the gel was placed on the top of the SDS-PAGE slab gel, and maintained in position with 1% agarose. The slab gel was run until bromophenol blue reached the bottom of the gel (An et al., 1988, 1989). The proteins were stained with Coomassie blue R-250 and destained.

Immunization procedures

Four 6-week-old Balb/cBYJ female mice were each immunized with 100 μg protein C freshly emulsified in RIBI adjuvant (Monophosphoryl Lipid A-Trehalose Dimycolate). The mixture was injected in 50 μL aliquots into each of four separate subcutaneous sites on the ventral side near the axillary and inguinal lymphatics, in 150 μL volume into the intraperitoneal cavity, and in 150 μL into one anterior dorsal subcutaneous site. The injection process was repeated three times, each at two-week intervals. The titer of the serum against protein "C" was determined by ELISA, as described below, one week after the second and third booster injections. A final boost was given intraperitoneally 26 days after the third booster and four days prior to the fusion.

Development of monoclonal anti-protein C antibodies

Monoclonal antibodies against protein C were produced using previously established protocols of Kao and Klein (1986). Spleen cells harvested from immunized mice were fused with SP2/0 myeloma cells at a ratio of 7.5:1 (spleen cells: myeloma cells) using 50% polyethylene glycol 1540. The fused cells were suspended in hypoxanthine-aminopterin-thymidine (HAT) selective medium and seeded into 96-well flat-bottom culture plates. Ten to 14 days later, the supernatants of growth-positive wells were screened for production of anti-protein C antibodies by testing with protein C and rock shrimp protein extracts by ELISA and immunodot blot. Hybridomas initially producing anti-protein C were expanded and rescreened. Hybridomas that showed continued production of anti-protein C antibodies were cloned by the limiting dilution method (Zola and Brooks, 1982). McAb isotypes were determined with ScreenType™ (Boehringer Mannheim Biochemicals).

Three cloned hybridomas (designated as 2E8-2B10, 4H1-8F11 and 4H2-10D3) that produced anti-protein C McAb with high reactivity for rock shrimp extract only were propagated intraperitoneally in three to four male Balb/cBYJ mice to produce McAb-containing ascites. Anti-protein C McAbs from these ascites were purified using the protein-A Sepharose 4B (Sigma) column chromatography (Ey et al., 1978).

Enzyme-linked immunosorbent assay for protein C

Each well of a 96-well Nunc immunoplate was coated overnight at 4°C with 100 μL (5 μg/mL) of protein C or water protein extracts in PBS containing 0.02% azide, pH 7.4 (PBS-az). Each well was then washed three times with PBS-az containing 0.5% Tween-20 (PBS-T) and incubated with 1% bovine serum albumin (BSA) in PBS for 1 hr at 4°C. The wells were washed three times again with PBS-T and incubated at room temperature for 1 hr with supernatants of the hybridoma cultures or purified McAb (4H2-10D3) in 100 μL/well. After three washes with PBS-T, the wells were added with the rabbit anti-mouse IgG conjugated with alkaline phosphatase (Sigma) and incubated for 1 hr. After three additional washes with PBS-T, p-nitrophenyl-phosphate (Sigma) was added to each well, and the plate incubated for 45 min in the dark. Absorbance was read at 405 nm using an ELISA plate reader.

Immunodot Blotting

Aqueous shrimp protein extracts (20 μg/20 μL) were dotted onto nitrocellulose membrane paper (BA85, Schleicher and Schuell) assembled in a Minifold I dot blot apparatus with an incubation plate, and incubated for 1 hr. The nitrocellulose membrane were then blocked by incubating with PBS-az containing 5% nonfat milk for 1 hr at room temperature. Following washing with PBS-T for 5 min, the membrane was incubated in the purified McAb (100 μg/mL) in PBS-az containing 1% BSA for 30 min. After washing for 5 min with PBS-T, the membrane was incubated with rabbit anti-mouse IgG (1 μg/mL) conjugated with alkaline phosphatase for 30 min. Following additional washing with PBS-T, the membane was incubated in nitroblue tetrazolium (0.1 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.05 mg/mL) solution in 0.1 M Tris buffer containing 1 mM $MgCl_2$ (pH 8.8) until blue color development.

Western blotting

Crude protein extracts of pink, white, and rock shrimp after electrophoresis on 12% SDS-PAGE gels (An et al., 1988) were electrophoretically blotted onto Immobilon membrane paper (Millipore Corp.) at 4°C using a Trans-Blot device (Bio-Rad) at 50 mA for 2 hr. After proteins were transferred onto the membrane, they were processed by the same techniques used for immunodot blotting with only slight modifications. The membrane paper was blocked with 5% nonfat milk in PBS-az for 2 hr, followed by 2 and 1 hr incubations in the respective solution containing the purified McAb and the alkaline phosphatase conjugate.

Pretreatment of antigens

Water extracts of rock, pink, and white shrimp were either heat-treated or maintained as the native state. Heat-treated samples were prepared by boiling the extracts in water for 5 min followed by centrifugation at 2000 x g for 5 min to remove protein aggregates. Both the native and heat-treated samples were analyzed for protein concentration using the Lowry method, diluted to 5 μg/mL, and then added with or without SDS (final concentration: 0.1% SDS). The samples were each reacted with the 4H2-10D3 McAb at 100 μg protein/mL on immunodot blot or by ELISA to determine if heat treatment or the addition of SDS would increase the reactivity of rock shrimp proteins with this McAb.

Optimization of McAb and antigen concentrations for ELISA

Antibody concentration. McAb at 0.5, 1, 5, 10, 50, 100, or 200 μg protein/mL was tested by ELISA to determine the optimal concentration needed to achieve highly specific reactivity for rock shrimp proteins. Only the heat-treated water extracts of rock, pink or white shrimp at 5 μg protein/mL were used to coat the plate wells.

Antigen concentration. Heat-treated water extracts of pink, white and rock shrimp at 0.5, 1, 5, 10, 50, 100, or 500 μg protein/mL were used to coat the plate wells (0.1 mL/well). 4H2-10D3 McAb at 50 or 100 μg protein/mL was tested on these plates to determine the optimal concentration of antigen needed for maximum reactivity in the ELISA test.

Blind study to detect and quantitate rock shrimp

A single blind test using ELISA was employed to determine the specificity of the 4H2-10D3 McAb for rock shrimp protein. The reactivity of rock shrimp was tested along with 23 other species of seafoods including oyster, scallop, lobster, clam, various fish and shrimp obtained from various locations, and meat samples including chicken, pork and beef. The water-soluble protein extracts of these diverse food samples were heat-treated for 5 min in boiling water followed by centrifugation. After protein concentration determination, each sample was adjusted to 10 μg/mL and a 100 μL aliquot was analyzed by ELISA. Experiments were performed in two replications.

The sensitivity of the 4H2-10D3 McAb in detecting rock shrimp in sample mixtures containing seafood and meat samples was conducted in a double-blind study using the ELISA test. The water extracts of the 26 seafood and meat samples (including 3 rock shrimp samples), after heat treatment and centrifugation, were each adjusted to a protein concentration of 500 μg/mL. They were combined randomly with each other or with PBS in various ratios unknown to the author to yield 24 sample mixtures (Table 1). The sample mixtures were serially diluted with PBS (1:5) to 5^{-7} of the original protein content. Each of the serially diluted samples at 100 μL was then subjected to the above mentioned ELISA test to determine the lowest amount of rock shrimp protein that reacted with the McAb. The most diluted samples which gave absorbance values greater than 0.2 were marked. The experiment was repeated once. The rock shrimp protein content in the marked samples was calculated using the following equation:

Rock shrimp content (ng) =
(Protein content, ng) x (Rock shrimp percentage) x (Dilution factor)

RESULTS AND DISCUSSION

SDS-PAGE banding patterns of various seafood and meat samples

Seafood species and meat samples each showed a distinct SDS-PAGE protein pattern useful for species identification (Fig. 1). The protein profile of rock shrimp was clearly different from those of the other seafoods and meats including clam, oyster, sea scallop, fish, beef, pork, and chicken. The two protein bands with mol wt 17700 and 18500 Da were unique for rock shrimp. They were referred to as protein C.

Comparison of the protein profiles of the various shrimp samples and lobsters collected worldwide revealed that the protein pattern of lobster was different from those of shrimp samples (Fig. 2). Pink, white, blue and brown shrimp in general had a relatively similar protein profile; the movement of the two major bands found in the lower one-third section of the gel were almost identical. When compared to rock shrimp protein C, these two bands had slightly lower R_f values and thus higher molecular weights.

Two-dimensional electrophoretic analysis showed that protein C was actually two proteins (data not shown). Protein C comprised 19.7% of the total water-soluble rock shrimp proteins as estimated from the intensity of the protein bands shown on the gel (data not shown).

Table 1. Composition of various unknown mixture samples used in Blind Study.

Sample No.	Percent composition (v/v) of various seafood and meat protein extracts[a]
Z1	50% TR + 50% SS
Z2	50% S2 + 50% B
Z3	50% S + 50% F
Z4	50% R2 + 50% PBS
Z5	50% R1 + 50% PBS
Z6	50% S7 + 50% S9
Z7	50% O + 50% TU
Z8	50% S10 + 50% L1
Z9	50% S5 + 50% C
Z10	50% G + 50% P
Z11	50% S3 + 50% L2
Z12	50% S8 + 50% S4
Z13	50% R3 + 50% PBS
Z14	33% CH + 33% S1 + 33% S6
Z15	33% R2 + 33% O + 33% SS
Z16	33% R2 + 33% C + 33% S10
Z17	33% R3 + 33% S4 + 33% S1
Z18	33% R3 + 33% CH + 33% L2
Z19	33% R1 + 33% S10 + 33% L1
Z20	17% R1 + 17% R2 + 17% R3 + 33% G + 17% PBS
Z21	17% TR + 17% SS + 17% S2 + 17% B + 33% R2
Z22	17% TR + 17% S + 17% F + 17% R1 + 17% S7 + 17% S9
Z23	17% O + 17% SS + 17% C + 17% S10 + 33% R2
Z24	29% R1 + 14% S5 + 29% P + 29% PBS

[a]The water extract was adjusted to 500 μg/mL.

Abbreviations designate: R1, rock shrimp from Port Canavarel, FL; R2, rock shrimp from Cedar Key, FL (February); R3, rock shrimp from Cedar Key, FL (December); S1, white shrimp from Ecuador; S2, Argentine-red shrimp from Argentine; S3, Blue shrimp from Ecuador; S4, pink shrimp from Tampa, FL; S5, white shrimp from Peru; S6, brown shrimp from North Carolina; S7, white shrimp from Columbia; S8, white shrimp from Georgia; S9, white shrimp from Honduras; S10, blue shrimp from Honduras; L1, lobster from Australia; L2, lobster from Florida; C, clam; O, oyster; SS, sea scallop; TR, trout; S, salmon; F, flounder; TU, tuna; G, grouper; B, beef; P, pork; CH, chicken.

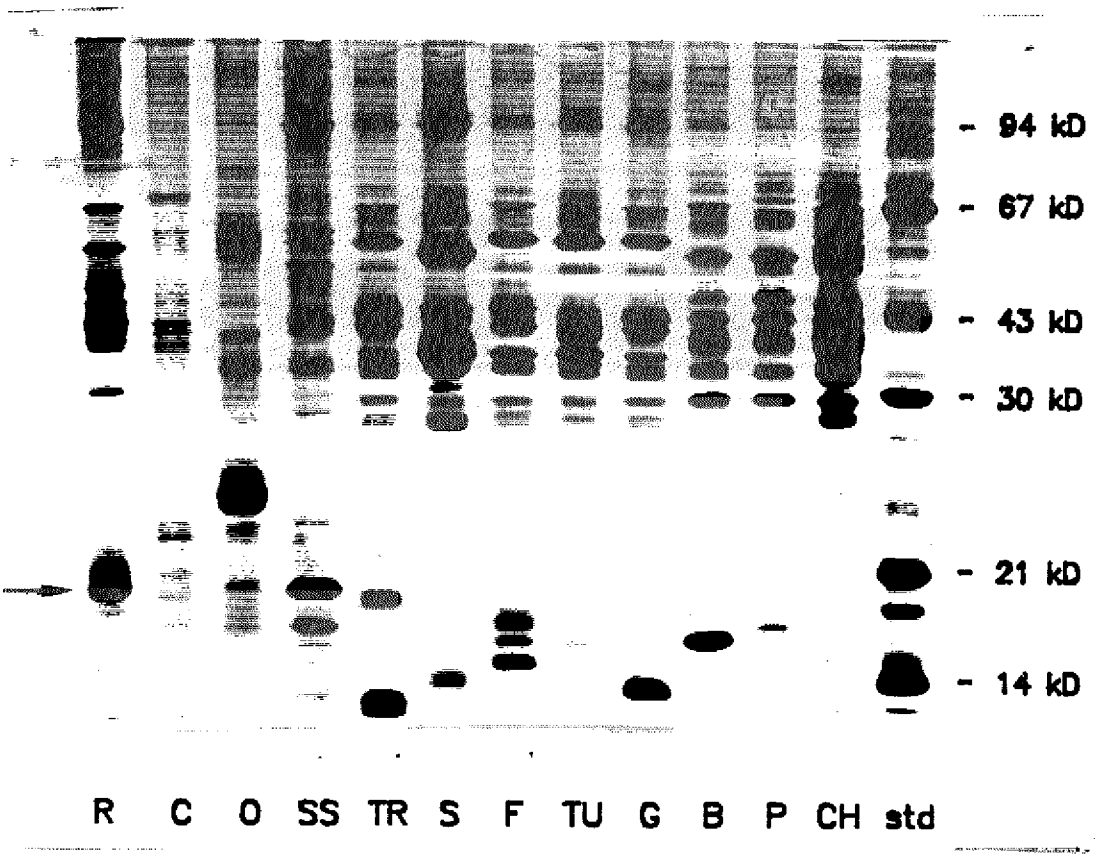

Figure 1. SDS-PAGE profiles of water-soluble proteins from various seafood and meat (cathode on top). The arrow designates protein C. R, rock shrimp from Cear Key, FL; C, clam; O, oyster; SS, sea scallop; TR, trout; S, salmon; F, flounder; TU, tuna; G, grouper; B, beef; P, pork; CH, chicken; std, low mol wt protein standards with mol wt marked on the side.

Screening of monoclonal anti-protein C antibodies

Eight of the original 400 seeded cultures were found to produce antibodies that showed 3 to 10 times higher reactivity for rock shrimp protein than for pink or white shrimp protein as determined by ELISA. They also showed high reactivity for protein C; the absorbance readings were > 0.4.

The results of the immunodot blot tests indicated that antibodies produced by these eight cultures failed to react with the water extracts of pink and white shrimp (Fig. 3); the antibodies only showed weak reaction with rock shrimp extract as indicated by the faint blue color developed. However, antibodies from the 2E8, 4H1 and 4H2 cultures showed positive reactions with rock shrimp extracts containing 0.1, 0.5 or 1.0% SDS. Based on color intensity, rock shrimp extract containing 0.1% SDS showed the highest reactivity, which was followed by 0.5 and 1.0% SDS (Fig. 3). Based on the ELISA and immunodot blot test results, these three cultures were subjected to cloning. Again the cloned cultures only showed high reactivity with rock shrimp extract containing 0.1% SDS by immunodot blot test. No reactivity occurred with pink or white shrimp extracts by ELISA or immunodot blot test (data not shown).

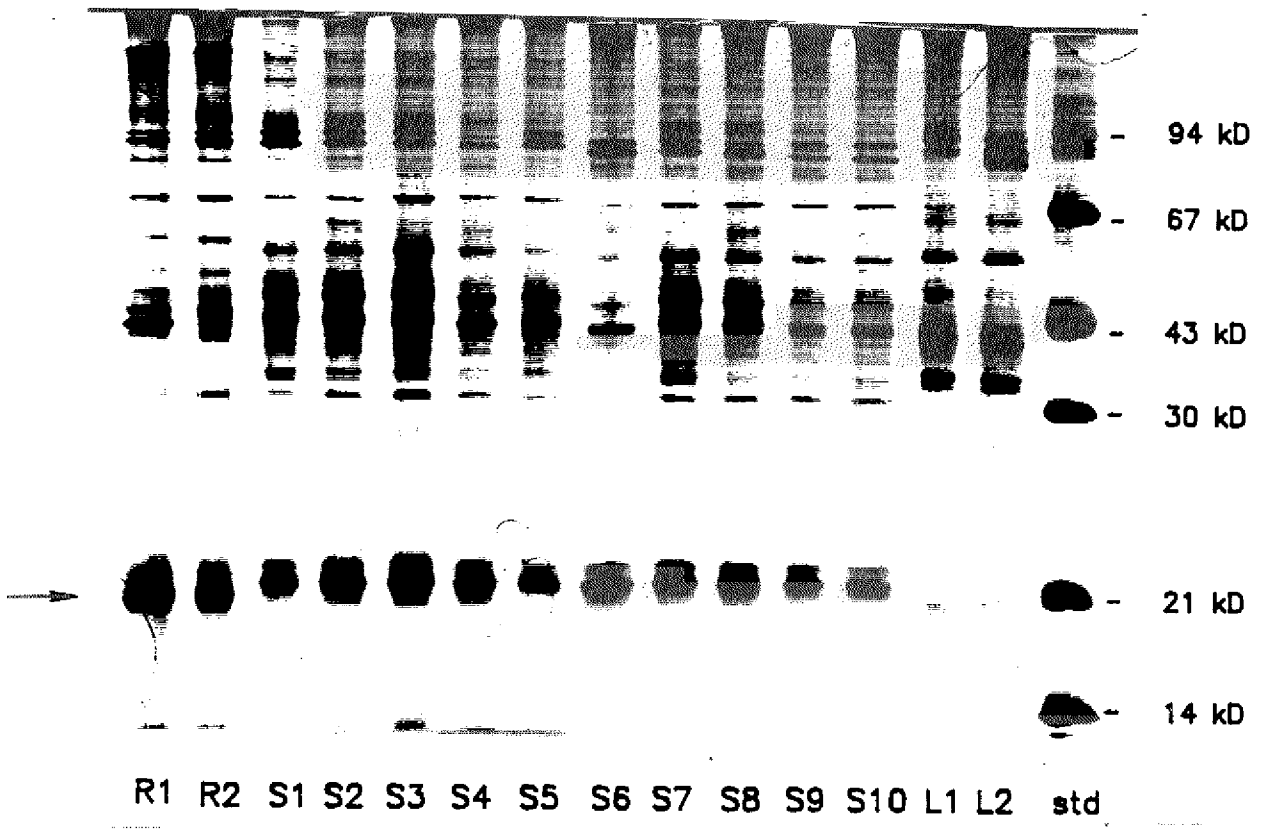

Figure 2. SDS-PAGE profiles of water-soluble proteins from various shrimp and lobsters (cathode on top). R1, rock shrimp from Fort Canavarel, FL; R2, rock shrimp from Cedar Key, FL; S1, white shrimp from Ecuador; S2, Argentine-red shrimp; S3, blue shrimp from Ecuador; S4, pink shrimp from Tampa, FL; S5, white shrimp from Peru; S6, brown shrimp from North Carolina; S7, white shrimp from Columbia; S8, white shrimp from Georgia; S9, white shrimp from Honduras; S10, blue shrimp from Honduras; L1, lobster from Australia; L2, lobster from Florida; std, low mol wt protein standards with mol wt marked on the side. Rock shrimp protein C also marked with an arrow.

The ELISA test results also showed that most clones, especially those derived from the 4H1 and 2E8 cultures, had much higher absorbance readings for protein C (0.41-2.0) than for rock shrimp extract (0.2-0.9). Since protein C was used to immunize the mice, the McAbs produced this way would be expected to have a higher reactivity for protein C than for rock shrimp extract. Heat treatment can cause protein denaturation and exposure of some antigenic sites that may not be evident when the protein is in its native form (Foegeding, 1988). Thus McAb specific for heat-induced structure may not react as well with the protein molecule in its native state (Benjamin et al., 1984).

Only one clone from each culture showing the highest reactivity for rock shrimp extract (2E8-2B10, 4H1-8F11 and 4H2-10D3) was chosen to propagate intraperitoneally in mice to produce McAb-containing ascites. All McAbs belonged to the $IgG_1(k)$ isotype as determined by the isotyping kit (data not shown). In the presence of ß-mercapthoethanol, the McAbs only showed two

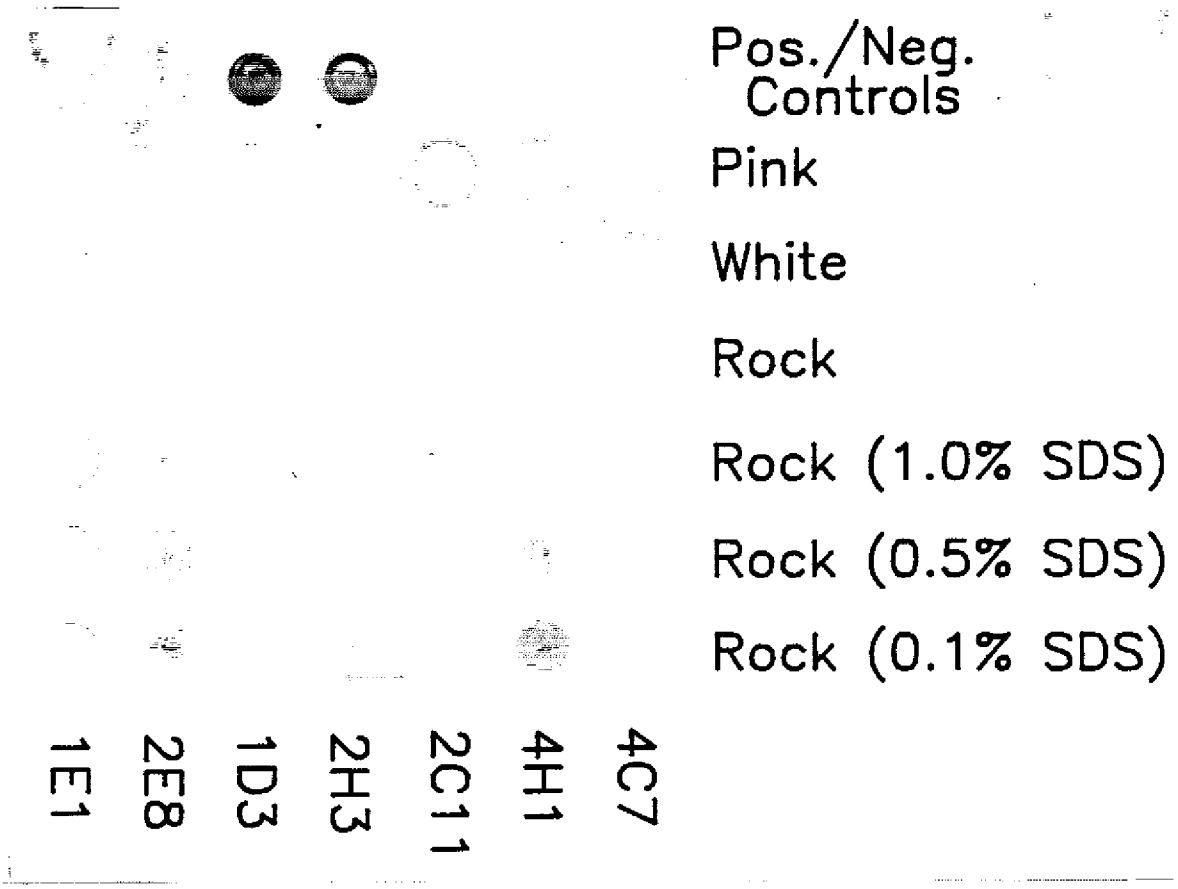

Figure 3. Screening of the uncloned hybridoma cultures by immunodot blotting for reactivity with rock shrimp extracts added with SDS. Pink and white shrimp were used as negative controls, while mouse IgG was used as positive control. Culture 4H2 which showed similar results as 4H1 and 2E8 was not included in this picture.

distinct bands (light and heavy chains) on the 12% polyacrylamide gels after SDS-PAGE. After purification by affinity chromatography, the McAbs were assessed again for reactivity with rock, pink and white shrimp extracts by immunodot blot; the 4H2-10D3 McAb showed the highest specific reactivity for rock shrimp extract (data not shown). This McAb was therefore used to develop immunochemical assays to detect rock shrimp.

Pretreatment of proteins for reaction using immunodot blot test

4H2-10D3 McAb was shown by immunodot blot test to react with rock shrimp extract in the native state. However, heat treatment of the extract greatly enhanced the reactivity with this McAb (Fig. 4). Since protein C used to immunize mice had been heat-denatured before the SDS-PAGE run, it is not surprising that 4H2-10D3 McAb is more reactive to heat-treated rock shrimp extract than to non-heated extract. The high specificity of this McAb for heat-denatured proteins enables its use to detect the presence of rock shrimp in processed seafood products.

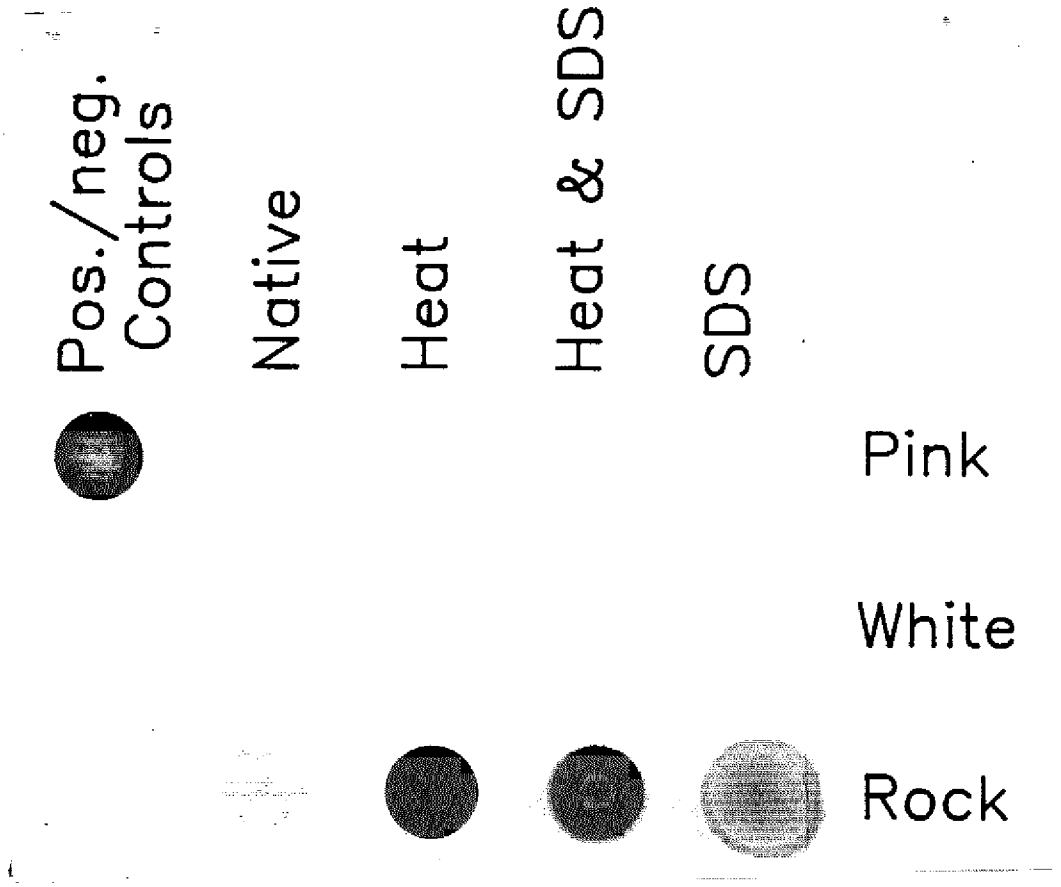

Figure 4. Effect of the heat treatment, addition of SDS or the combination of both on the reactivity of rock shrimp extract with 4H2-10D3 McAb as analyzed by immunodot blot. Mouse IgG and BSA were used as positive and negative controls.

The addition of SDS to heat-treated or non-treated rock shrimp extract also increased antigen reactivity with the 4H2-10D3 McAb on the immunodot blot, but to a lesser extent than heat treatment of the antigen alone (Fig. 4). The increase in protein reactivity in the presence of low concentration of SDS was believed to be related to protein conformational changes caused by SDS. SDS might also reduce the amount of heat-denatured proteins available for reaction with McAb by affecting the binding of the former to the nitrocellulose membrane. Therefore, protein samples were heat treated for immunodot blot tests in later studies.

Specificity determination of 4H2-10D3 McAb by Western blotting and immunodot blotting

McAb 4H2-10D3 showed only one positive reaction area that corresponded to protein C of rock shrimp on immunostained blot, while no reaction occurred for pink and white shrimp. This result indicated that the 4H2-10D3 McAb reacted only with the protein C component of rock shrimp and not with the proteins from pink or white shrimp, or with the protein standards.

To determine if any cross-reactivity occurred, the 4H2-10D3 McAb was tested by immunodot blot against 23 commonly consumed unrelated species of seafood and

meat (clam, oyster, sea scallops, chicken, pork, beef, fishes, lobsters, 10 different shrimp from various regions) as well as the three rock shrimp harvested from the East and West coasts of Florida (Port Canaveral and Cedar Key) or at two different seasons, February and December. The McAb showed similar reactivity with rock shrimp samples harvested from different locations (dots A2 and A3 in Fig. 5a; and C1 and D1 in Fig. 5b) and during different seasons (dots A3 and C7 in Fig. 5a), indicating that this McAb was highly specific for rock shrimp regardless of the harvest location and season. No cross-reactivity was observed with the other shrimp species or lobsters. This rock shrimp-specific McAb can thus be used for effective shrimp differentiation purposes.

The 4H2-10D3 McAb reacted slightly with the distant species including tuna, beef, pork and chicken, as indicated by the light color developed. Protein C-like molecules may thus present in these distant species. However, the intense blue color developed for rock shrimp was easily differentiated from the less reactive species. The 4H2-10D3 McAb is thus highly specific for rock shrimp.

Optimization of ELISA test

Pretreatment of antigens. Heat treatment of rock shrimp extract in boiling water for 5 min enhanced the reactivity with the 4H2-10D3 McAb using ELISA test (Fig. 6). However, the addition of SDS to heat-treated or non-treated rock shrimp extract decreased the antigenic reactivity with the McAb (Fig. 6). Protein binding to plastic microtitration plates is achieved by adsorption through hydrophobic interactions between nonpolar protein substructures and the nonpolar plastic matrix (Clark and Engvall, 1980). The presence of interfering chemicals, such as SDS, can affect this weak binding (Engvall, 1980). Therefore, the adsorption of shrimp antigen onto ELISA plates may have been reduced, and resulted in a reduced reactivity with the McAb.

Antibody concentration. The reactivity of the 4H2-10D3 McAb with rock shrimp extract at 5 μg/mL increased as the amount of McAb used was increased; the reactivity plateaued when antibody concentration reached 20 μg/mL (Fig. 7A). To ensure high reactivity, McAb at 50 and 100 μg/mL was used to determine the optimal antigen concentration for reaction.

Antigen concentration. Rock shrimp extract at 10 μg/mL showed the highest reactivity with the 4H2-10D3 McAb at 50 or 100 μg/mL (Fig. 7B). The limited binding sites on the plastic surface for antigen could account for the "prozone" effect. The surface binding sites approaches saturation when a high concentration of antigen was added (Douillard and Hoffman, 1983; Pesce et al., 1981). In addition, proteins at high concentration may aggregate and reduce the number of antigenic determinants available for antibody binding. For a better ELISA result, rock shrimp extract as well as the unknown samples at 10 μg/mL was used to react with the McAb.

The use of McAb at 50 μg/mL generally produced a lower absorbance reading than at 100 μg/mL (Fig. 7B). This effect was more significant at an antigen level of less than 10 μg/mL. McAb binding to antigen-coated immunosorbent matrix has been reported to be proportional to antibody concentration (Pesce et al., 1977); thus the more concentrated the McAb, the better the McAb binds to the antigen coated on the plate. McAb at 100 μg/mL was used for ELISA test to ensure the most effective detection and quantitation of rock shrimp.

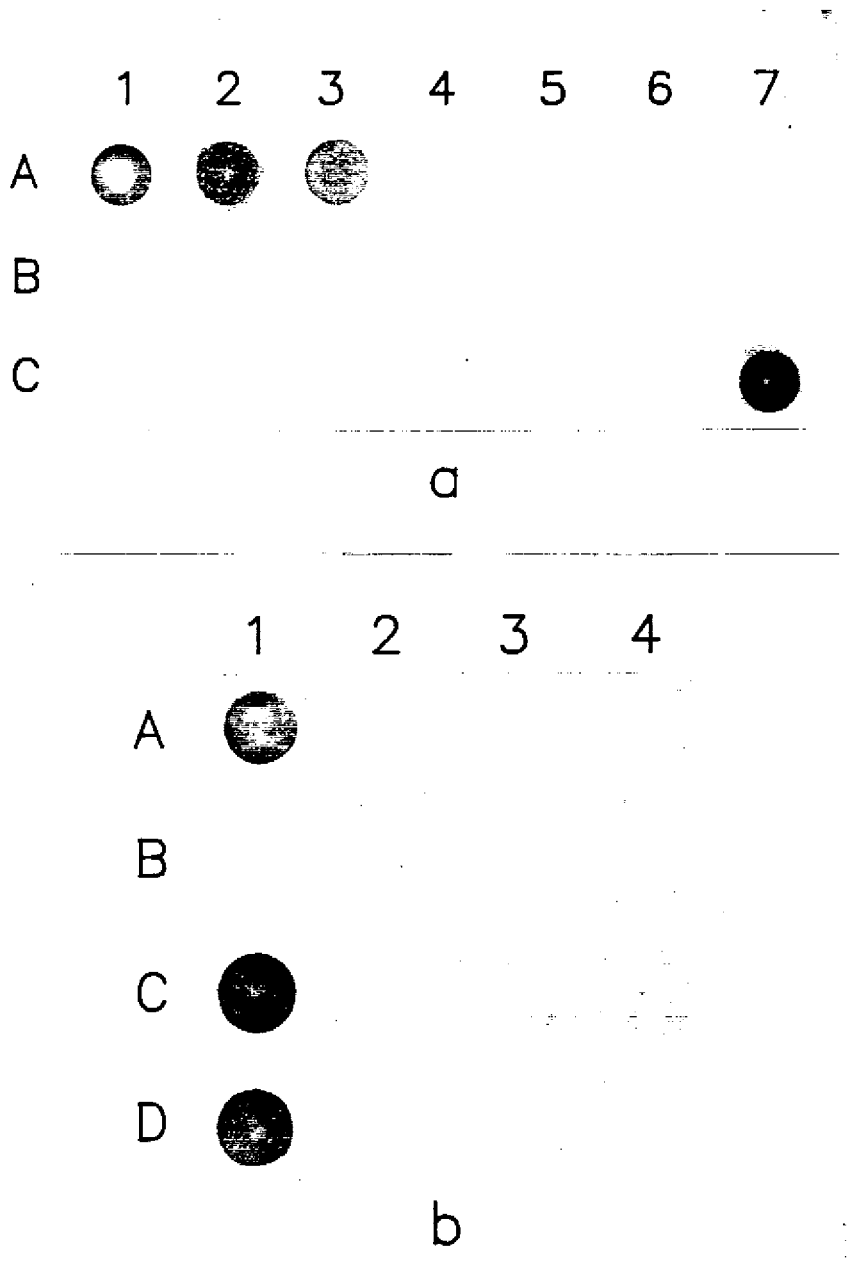

Figure 5. Determination of the specific reactivity of 4H2-10D3 McAb with rock shrimp as analyzed on immunodot blots with the water extracts in 20 μL containing 20 μg of proteins: (a) various shrimp and lobsters (b) various seafood and meat. Samples used in test (a) include A1, mouse IgG; B1, 1% BSA; A2, rock shrimp from Port Canavarel, FL; A3, rock shrimp obtained in February from Cedar Key, FL; A4, white shrimp from Ecuador; A5, Argentine-red shrimp; A6, blue shrimp from Ecuador; B2, pink shrimp from Tampa, FL; B3, white shrimp from Peru;B4, brown shrimp from North Carolina; B5, white shrimp from Columbia; B6, white shrimp from Georgia; C2, white shrimp from Honduras; C3, blue shrimp from Honduras; C4, Lobster from Australia; C5, Lobster from Florida; C7, rock shrimp obtained in December from Cedar Key, FL. Samples used in test (b) include A1, mouse IgG as positive control; B1, 1% BSA as negative control; C1, rock shrimp from Fort Canaveral, FL; D1, rock

shrimp from Cedar Key, FL; A2, clam; B2, oyster; C2, sea scallop; D2, trout; A3, salmon; B3, flounder; C3, tuna; D3, grouper; A4, beef; B4, pork; C4, chicken.

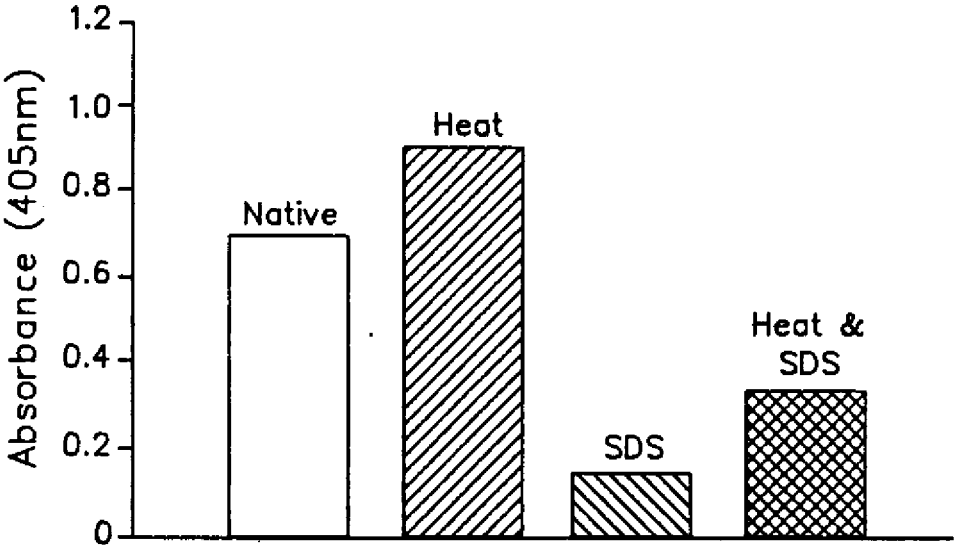

Figure 6. Effect of heat treatment, the addition of SDS or the combination of both on the increment of reactivity of rock shrimp proteins at 5 μg/mL with 4H2-10D3 McAb on ELISA.

Identification of rock shrimp in a blind study

Rock shrimp samples obtained from the East and West coasts of Florida (samples X8 and X7) and at different months (February and December; samples X7 and X22) were equally reactive with the 4H2-10D3 McAb and were correctly identified in a blind study using 24 various seafood and meat samples (Table 2). The reactivity of this McAb for rock shrimp samples was 9 times higher than that for the other seafood and meat samples, or the other unrelated shrimp samples.

Detectability of the ELISA test for rock shrimp in mixtures in a blind study

Among the 24 unknown samples prepared by randomly mixing the various seafood, fish or meat samples in various ratios with rock shrimp (Table 1), all 13 sample mixtures containing rock shrimp were correctly identified using the test conditions developed for ELISA. These positive samples all had an absorbance reading greater than 1.0 (data not shown).

The reactivity of the McAb to detect the lowest amount of rock shrimp present in these positive mixture samples was determined. The detectability was interpreted as the minimal amount of rock shrimp protein present in the most

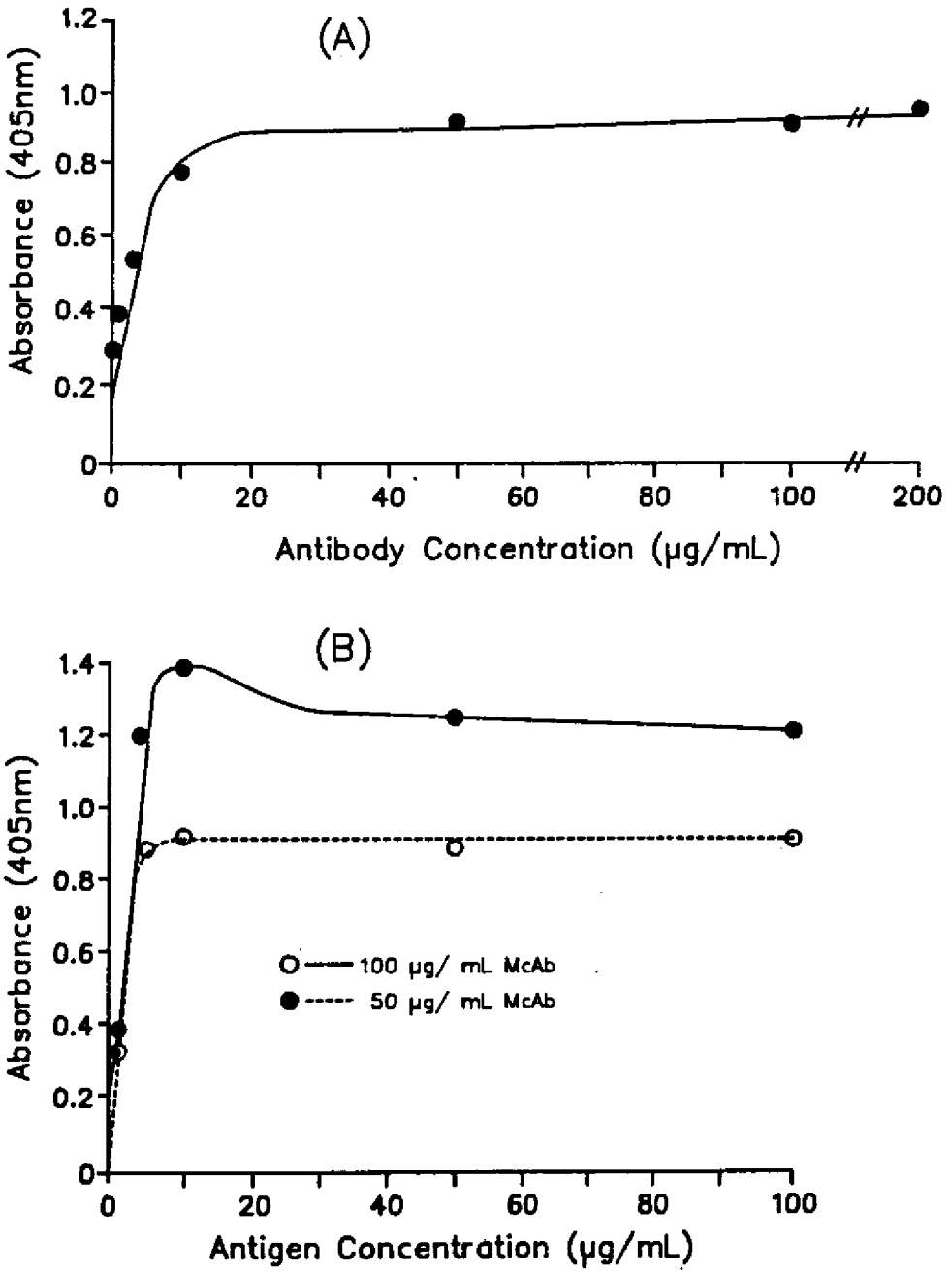

Figure 7. Dose-related changes of the reactivity of 4H2-10D3 McAb with rock shrimp extract determined by ELISA. (A) Rock shrimp extract at 5 µg/mL was reacted with different amount of McAb. (B) McAb at 50 and 100 µg/mL was reacted with different amount of rock shrimp extract.

Table 2. The reactivity of the 4H2-10D3 McAb[a] with the water extracts[b] of the various seafood and meat samples in the blind study using the ELISA test.

Sample No.	Unknown sample	Optical density[c]
X1	Trout	0.111 ± 0.012
X2	Sea scallops	0.095 ± 0.009
X3	Argentine-red shrimp, Ecuador	0.095 ± 0.011
X4	Beef	0.097 ± 0.004
X5	Salmon	0.126 ± 0.002
X6	Flounder	0.102 ± 0.008
X7	Rock shrimp, Cedar Key, FL (February)	1.508 ± 0.068
X8	Rock shrimp, Port Canaveral, FL	1.125 ± 0.071
X9	White shrimp, Columbia	0.112 ± 0.016
X10	White shrimp, Honduras	0.109 ± 0.006
X11	Oyster	0.098 ± 0.012
X12	Tuna	0.117 ± 0.002
X13	Blue shrimp, Honduras	0.093 ± 0.010
X14	Lobster, Australia	0.094 ± 0.011
X15	White shrimp, Peru	0.099 ± 0.015
X16	Clam	0.096 ± 0.004
X17	Grouper	0.102 ± 0.008
X18	Pork	0.098 ± 0.007
X19	Blue shrimp, Ecuador	0.097 ± 0.011
X20	Lobster, FL	0.094 ± 0.011
X21	Brown shrimp, NC	0.097 ± 0.010
X22	Rock shrimp, Cedar Key, FL (December)	1.303 ± 0.059
X23	Pink shrimp, FL	0.096 ± 0.012
X24	Chicken	0.104 ± 0.019
X25	White shrimp, Ecuador	0.112 ± 0.013
X26	White shrimp, GA	0.106 ± 0.014

[a] The McAb used was 10 μg protein/well.

[b] The protein extracts used was 1 μg/well.

[c] Data are means ± standard deviations.

diluted samples that, after reacting with McAb, had an absorbance reading greater than 0.2 which was at least two times greater than the readings for background and negative controls. The detectability was found to range from 0.19 to 13.6 ng with an average of 4.3 ng (Table 3). The variation of the values was partially attributed to the numerical variation of the absorbance readings that were used for the determination of the rock shrimp levels in these sample mixtures. Rock shrimp proteins in highly diluted samples bound better to the microtitration plates, perhaps due to the reduced competition by other proteins for the same binding sites. The composition of the sample mixtures containing seafood and/or meat proteins also could affect the reactivity of rock shrimp extract with the McAb.

Protein C accounted for 19.7% of total rock shrimp protein; therefore the sensitivity of using the 4H2-10D3 McAb in detecting protein C in ELISA test was 0.84 ng (4.3 ng X 19.7%). This ELISA assay could be used to reveal the presence of rock shrimp at nanogram levels in mixtures containing unrelated species of seafood or meat.

Table 3. Sensitivity limit of the ELISA test in determining rock shrimp content in mixture samples.

Positive samples[a]	Absorbance	Dilution range	Protein content (μg/mL)	Rock shrimp percentage	Rock shrimp content[b] (ng/well)
Z4	0.229	5^{-7}	250	100	0.32
Z5	0.219	5^{-5}	250	100	8
Z13	0.325	5^{-5}	250	100	8
Z15	0.342	5^{-7}	500	33	2.1
Z16	0.295	5^{-5}	500	33	5.3
Z17	0.278	5^{-5}	500	33	5.3
Z18	0.281	5^{-5}	500	33	5.3
Z19	0.203	5^{-6}	500	33	1.1
Z20	0.263	5^{-6}	417	60	1.6
Z21	0.250	5^{-6}	333	33	0.7
Z22	0.393	5^{-4}	500	17	13.6
Z23	0.263	5^{-7}	500	30	0.19
Z24	0.303	5^{-5}	333	40	4.2

[a] Positive mixture samples containing rock shrimp as determined in the blind study by ELISA.

[b] Rock shrimp content was calculated by multiplying protein content with the dilution range and rock shrimp percentage in the mixture.

CONCLUSION

In summary, the 4H2-10D3 McAb developed in this study was proven to be rock shrimp-specific by ELISA, immunodot blot and Western blot tests. This McAb was reactive to heat-treated rock shrimp proteins, and was shown to be very effective and sensitive in blind studies to identify rock shrimp, and to detect the presence of rock shrimp in samples containing mixtures of diverse seafoods or other non-seafood meats using the ELISA test. The detection of rock shrimp proteins in various seafood and meat sample mixtures can be achieved at a level as low as 4.3 ng on the average using the optimal ELISA testing conditions.

ACKNOWLEDGEMENT

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USE OF A UREA GEL ISOELECTRIC FOCUSING TECHNIQUE FOR QUANTITATION OF SHRIMP IN SHRIMP-SURIMI MIXTURES

T.S. Huang, J.S. Chen, W.S. Otwell, M.R. Marshall and C.I. Wei

Food Science and Human Nutrition Department, University of Florida
Gainesville, Florida 32611

INTRODUCTION

Due to its light color, bland odor and unique gelling properties, surimi is combined with natural shellfish meat, shellfish flavoring agents, salt, water and starch, and/or egg white for the manufacture of a variety of fabricated seafood products such as crab legs, scallops, lobster and shrimp analogs (Lanier, 1986; Lee, 1986; Regenstein and Lanier, 1986). To meet Food and Drug Administration (FDA) guidelines, the fabricated seafood products must be labeled properly to include information on the fish species used as the main ingredient, and the other species, such as snow crab meat for crabmeat analog, used as the additional ingredient.

There are problems in enforcing accurate labelling of the specific seafood component and content in fabricated products, mainly due to the lack of a reliable analytical method. Products with claims of 35% crabmeat are widely sold when the use of over 10% crabmeat is known to show detrimental effects to the product (Regenstein and Lanier, 1986). The establishment of useful methods to correctly determine the content of the specific seafood components in fabricated surimi products is therefore of great need for regulatory purposes.

Isoelectric focusing (IEF) has been extensively used for seafood species identification (Lundstrom, 1980). Hamilton (1982), using thin layer polyacrylamide gel IEF, identified various fish species. Recently, Wei et al. (1989) and An et al. (1989) used the modified urea gel IEF to identify raw pink, white and rock shrimp species. The present study was carried out to investigate if a reliable method could be established using this modified urea gel IEF system to quantitate the weight content of a minor component in a surimi mixture, using the Alaska pollock surimi-pink shrimp mixture as a model system. Since the protein content in the aqueous supernatants of the water homogenates of surimi samples was low, efforts were also made to determine if the use of ultrafiltration system to concentrate proteins would affect the protein patterns. The effect of using two different homogenization procedures on protein patterns was also investigated.

MATERIALS & METHODS

Samples and protein extractant

Pink shrimp (*Penaeus duorarum*) were harvested and transported within 48 hr to the Food Science and Human Nutrition Department, University of Florida, Gainesville. They were stored at -33°C until needed. Shrimp were then thawed under tap water, peeled, and deveined.

Alaska pollock (*Theragra chalcogramma*) surimi was obtained from the Alaska Fisheries Development Foundation. Surimi was cut into small pieces of about 80 g, put in plastic bags, and stored at -33 °C until needed.

Water containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% (w/v) sodium azid was used to extract proteins from raw samples of surimi, shrimp, and surimi-shrimp mixtures.

Effect of different homogenization methods on sample protein patterns

Two homogenization procedures were compared for their effectiveness in extracting sample proteins for IEF runs. The first method included the use of a food processor (Presto MinnieMax Compact Food Processor) and a Polytron (Brinkmann Instrument), while the second only used the Polytron. In the first study, samples of surimi only, and surimi mixed with 5 or 10 % (w/w) raw pink shrimp at 80 g were each mixed with 2% NaCl and then blended in a 6°C cold room using the food processor until a shiny paste was formed. A paste sample (7 g) was removed, mixed with 21 mL aqueous extractant, and then subjected to homogenization at room temperature for 1 min using the Polytron (setting 9.2). In the second study, after the various surimi samples at 7 g were each added with 2% NaCl in separate beakers and chopped into small pieces, they each were combined with the aqueous solvent at a ratio of 1:3 (w/v) and then homogenized at room temperature for 1 min using a Polytron (setting 9.2). The homogenates prepared by either method were centrifuged at 26,900 x g for 20 min at 5°C. The supernatants were collected and the protein concentration determined (Lowry et al., 1951). They were then subjected to IEF runs at 50 μg total protein/well. This experiment was repeated three times.

Effect of protein concentration using ultrafiltation membrane on protein retention and IEF banding patterns

Samples of surimi, and surimi-pink shrimp mixtures after homogenization were centrifuged at 26,900 x g for 20 min. Ten milliliter portions of the supernatant of each sample was subjected to concentration at room temperature using a Filtron Omegacell unit equipped with a 10 K nominal molecular weight limit filter membrane (Pharmacia LBK Biotechnology Inc.) at a nitrogen pressure of 25 psi. After the volumes and protein concentrations of concentrate and filtrate of each sample were measured, the percent loss of protein in filtrates was determined. The IEF protein patterns of the concentrates (50 μg/well) were compared with those of non-concentrated supernatants (also at 50 μg/well). This experiment was repeated six times.

Isoelectric focusing

The gel mixture containing 4% (w/v) acrylamide, 2% (w/v) Triton X-100, 9.2 M urea, and an ampholyte mixture consisted of 20% pH 3-10 and 80% pH 4-6.5 at a final concentration of 6.2% (v/v) was used (Wei et al., 1989). Following the previously described preparation procedures (An et al., 1989), protein samples (60 μg/well) were applied to the gel, focused, and then stained with Coomassie blue R-250 (An et al., 1989).

Apparent pI values of the shrimp and surimi proteins were determined indirectly by comparing their R_f values on the gel with those of the protein standards following the method of An et al. (1989). The protein standards (Broad pI kit, pH 3-10, Pharmacia) contained: trypsinogen, pI 9.30; lentil lectin-basic band, pI 8.65; -middle band, pI 8.45;, -acidic band, pI 8.15; horse myoglobin-basic band pI 7.35, -acidic band, pI 6.85; human carbonic anhydrase B, pI 6.55; bovine carbonic anhydrase, pI 5.85; ß-lactoglobulin A, pI 5.20; soybean trypsin inhibitor, pI 4.55; and amyloglucosidase, pI 3.50.

Establishment of an equation for determining the percent content by weight of shrimp in surimi-shrimp mixtures

Samples of surimi only, surimi mixed with 2.5, 5.0, 7.5, 10.0 and 15.0% (w/w) of shrimp, and shrimp only at 7 g were each mixed with 2% NaCl and then 21 mL aqueous extractant. After the samples were Polytron homogenized, 10 mL of the supernatants were concentrated using ultrafiltration membrane as previously described. Following determination of the protein concentration, the concentrates were diluted to 5 $\mu g/\mu L$; samples at 60 μg were then applied together with protein standards to the gel and subjected to IEF runs. The gels, following staining and destaining, were scanned using a Bio-Rad Video Densitometer (model 620) at a transmission mode. The densitometer was connected to a Zenith personal computer and the output data for each sample were processed using the Bio-Rad 1-D Analyst Software. Each sample was scanned at five different positions along the length of the band, and the average of the five readings was used for constructing the mathematical relationship between the protein contents and the peak area readings of the specific band in these samples. Two surimi-specific (pI values 7.11 and 7.17), and two shrimp-specific bands (pI values 5.46 and 5.52) were used for the construction of the protein content - peak area relationships. The light source of the densitometer was a fluorescent cool white tube; the width of the transmitted light was 125 μm.

To determine the reliability of the mathematical model, blind studies were conducted. The surimi-shrimp mixture with a certain percent content by weight of shrimp unknown to the person operating the assay, was analyzed together with samples of surimi only, shrimp only, and standard surimi-shrimp mixtures as previously described. After the sample concentrates were adjusted to 5 $\mu g/\mu L$, they were subjected to IEF runs at 60 μg/well. The stained gels were then scanned using the video densitometer. Each sample was scanned five times at different positions. The averages of the readings of the two shrimp-specific (pI values 5.46 and 5.52) and surimi-specific bands (pI 7.11 and 7.17) were respectively used together with the protein contents of these specific bands in each sample, to construct the mathematical equation. Using this equation and the densitometer readings of the same bands in unknown samples, the percent content by weight of shrimp in the unknown sample was calculated.

RESULTS & DISCUSSION

Effect of different homogenization method on sample protein patterns

During the preparation of fabricated seafood products, surimi is usually mixed with salt and then blended to a shiny paste before minor ingredients, such

as shrimp and crabmeat, are added. The comparison of the use of only a Polytron for homogenization with the use of a food processor together with a Polytron revealed that the IEF protein patterns of the surimi, and surimi mixed with 5 or 10% pink shrimp were not affected by the treatment method used (Fig. 1). Since all the major surimi protein bands (with pI values of 5.03, 5.13, 5.32, 5.37, 5.55, 5.69, 5.75, 5.86, 6.14, 6.40, 6.43, 6.49, 6.63, 6.71, 6.78, 6.85, 6.95, 7.0, 7.03, 7.09, 7.11, and 7.17) and shrimp bands (with pI values of 5.30, 5.42, 5.46, 5.50, 5.52, 6.0, 6.03, 6.33, 6.67, 6.75, 6.80, 6.81, and 6.91) were present in samples subjected to either treatment method, the samples used in the following studies were only treated with polytron homogenization.

Effect of ultrafiltration process on protein retention and IEF patterns

The use of ultrafiltration for concentration of the supernatants did not alter the IEF protein patterns of the supernatants of the three tested samples (Fig. 2). The major surimi- and shrimp-specific protein bands present in supernatants were also present in the concentrates as shown on the IEF gels. About 4.08 ± 0.76 (mean ± standard deviation), 6.08 ± 1.13, and 5.20 ± 0.86% of the total protein in the supernatants were present in the filtrates after ultrafiltration of the surimi, and surimi mixed with 5 or 10% shrimp, respectively. Because the use of ultrafiltration membrane for concentration did not change the IEF protein patterns and caused only 3.7 to 6.2% loss of total proteins, this method was used to increase sample protein concentration for IEF runs. The inclusion of this concentration process is especially important for surimi or surimi samples containing low levels of shrimp. In these samples, the protein concentration in unconcentrated supernatants could be less than 5 $\mu g/\mu L$. When these supernatants in large volumes were used for IEF runs, proteins were usually not satisfactorily focused.

Establishment of an equation to correlate the relationship between specific band protein contents and peak areas

Attempts were made to correlate the peak area readings of the protein bands obtained by densitometric scanning of the gel with the protein contents of that specific band in standard surimi-shrimp mixtures (Fig. 3). Because the thickness of the protein bands was not evenly distributed along the entire length of the band, the use of the peak area readings obtained from just one densitometric scanning of the gel was not sufficient in determining accurate protein content. Thus each sample was scanned five times at five different positions, and the average of the five readings was used to construct the linear relationship. Using this approach, two shrimp-specific bands (pI 5.46 and 5.52) and two surimi-specific bands (pI 7.11 and 7.17) showed high correlation in terms of peak area readings and band protein contents (coefficiency range: 0.83 - 0.97). The protein content (P) of a shrimp-specific band in the mixture could be calculated using the following equation:

$$P = \frac{A \times C \times D \times E}{A \times C + B \times (1-C)}$$

where A is the shrimp protein content in shrimp supernatant, B is the surimi protein content in surimi supernatant, C is the percent content of shrimp by

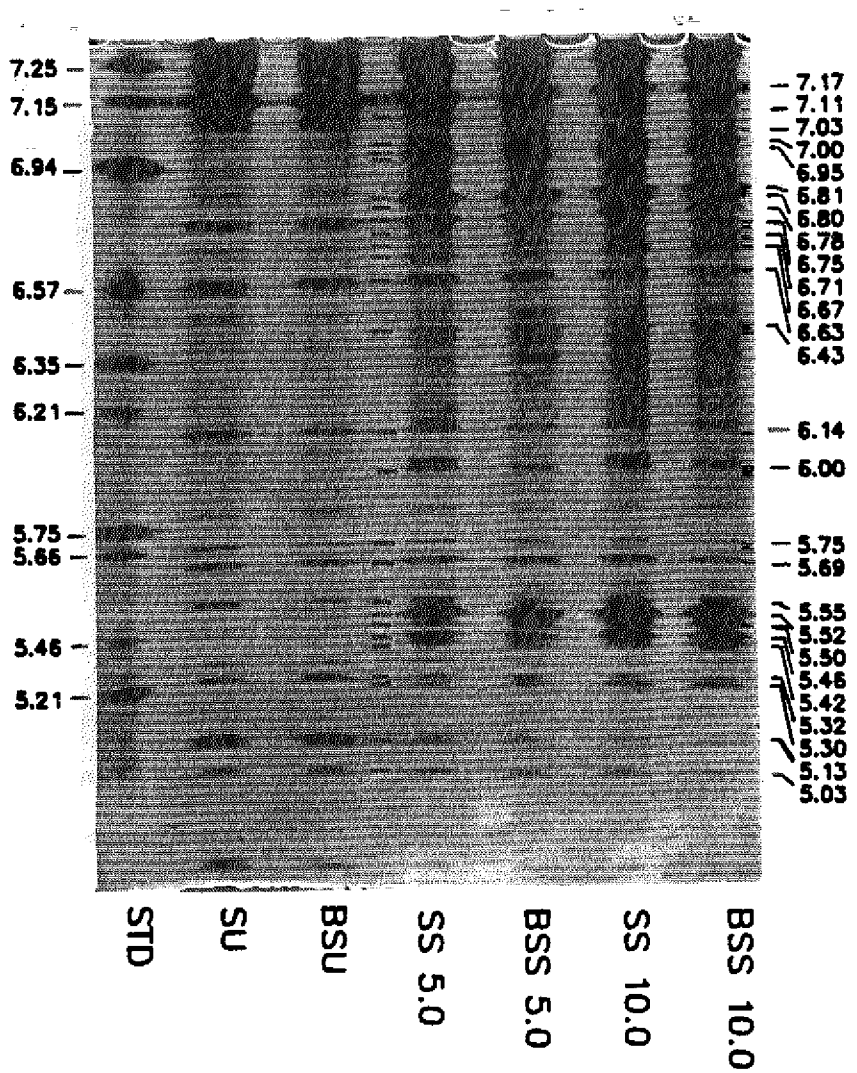

Figure 1. Comparison of the use of Polytron homogenization or food processor blending together with Polytron homogenization on the IEF protein patterns of the water extracts of surimi, and surimi mixed with 5 or 10% pink shrimp by weight (cathode on top). Protein standards (STD) are also included. The numerical values indicate apparent pI values of the protein bands. SU: surimi subjected to Polytron homogenization; BSU: surimi subjected to food processor and Polytron homogenization; SS 5.0 and SS 10.0: Polytron homogenization of surimi-shrimp mixtures containing 5 or 10% shrimp; BSS 5.0 and BSS 10.0: food processor plus Polytron homogenization of surimi-shrimp mixtures containing 5 or 10% shrimp.

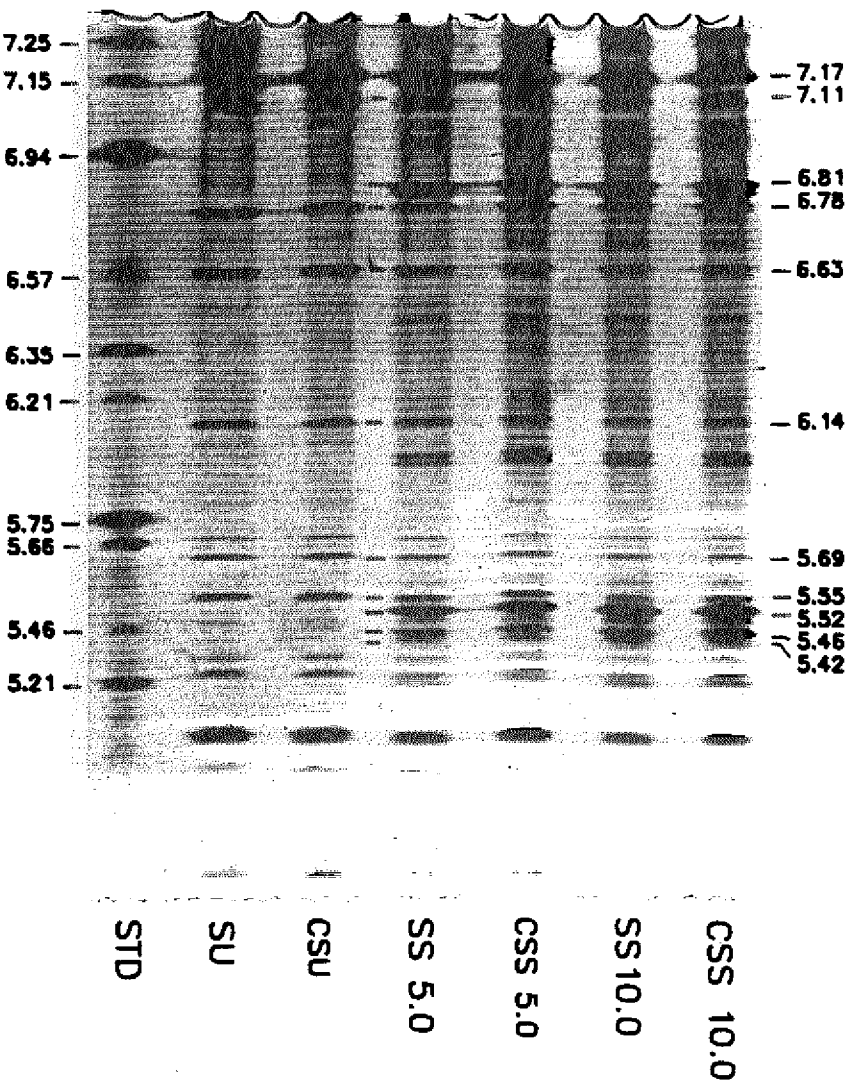

Figure 2. IEF patterns of the water extracts of surimi, and surimi-pink shrimp mixtures containing 5 or 10% shrimp before and after concentration by ultrafiltration membrane. Protein standards (STD) are also included (cathode on top). The numerical values indicate apparent pI values of the protein bands. SU and CSU: surimi extracts before and after concentration; SS 5.0 and SS 10.0: water extracts of surimi-shrimp mixtures containing 5 or 10% shrimp; CSS 5.0 and CSS 10.0: concentrates of the water extracts of surimi-shrimp mixtures containing 5 or 10% shrimp.

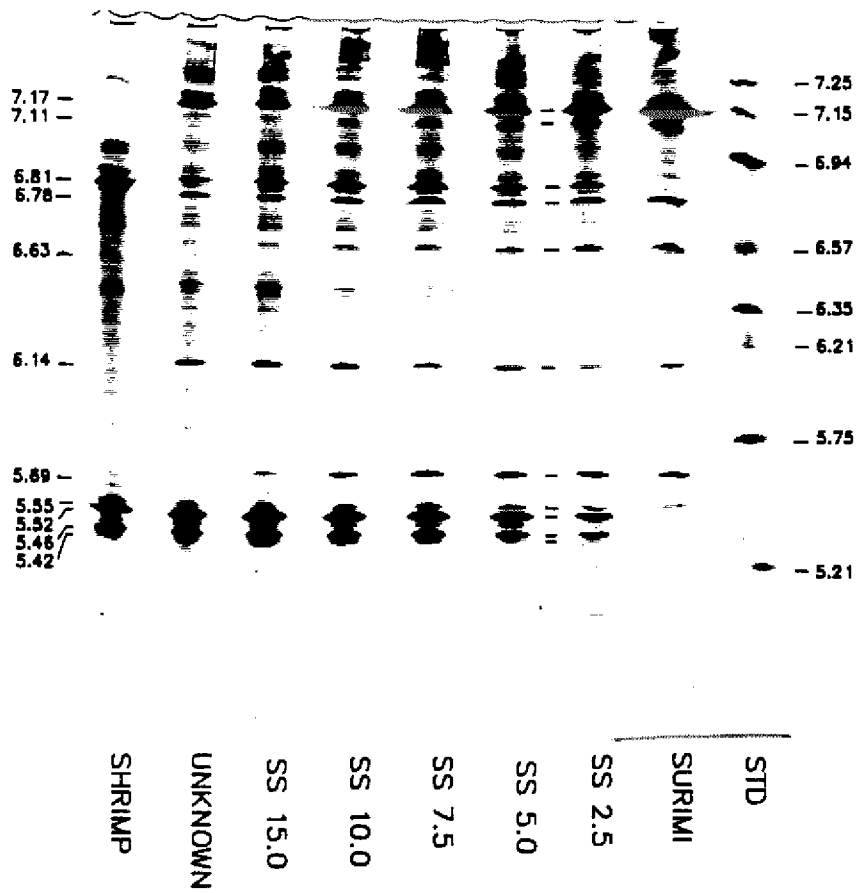

Figure 3. IEF patterns of water extracts of surimi, raw pink shrimp, and surimi-pink shrimp mixtures containing different percent by weight of shrimp (cathode on top). Protein standards (STD) are also included. The numerical values indicate apparent pI values of the protein bands. SS: surimi-pink shrimp mixture.

weight in surimi-shrimp mixture (2.5 or 5.0%, etc.), D is the percent of the specific shrimp protein band of interest as determined by densitometric scanning of the shrimp protein profile on the IEF gel (Fig. 4), and E is the total amount of protein applied in each well for IEF run (60 μg in this study).

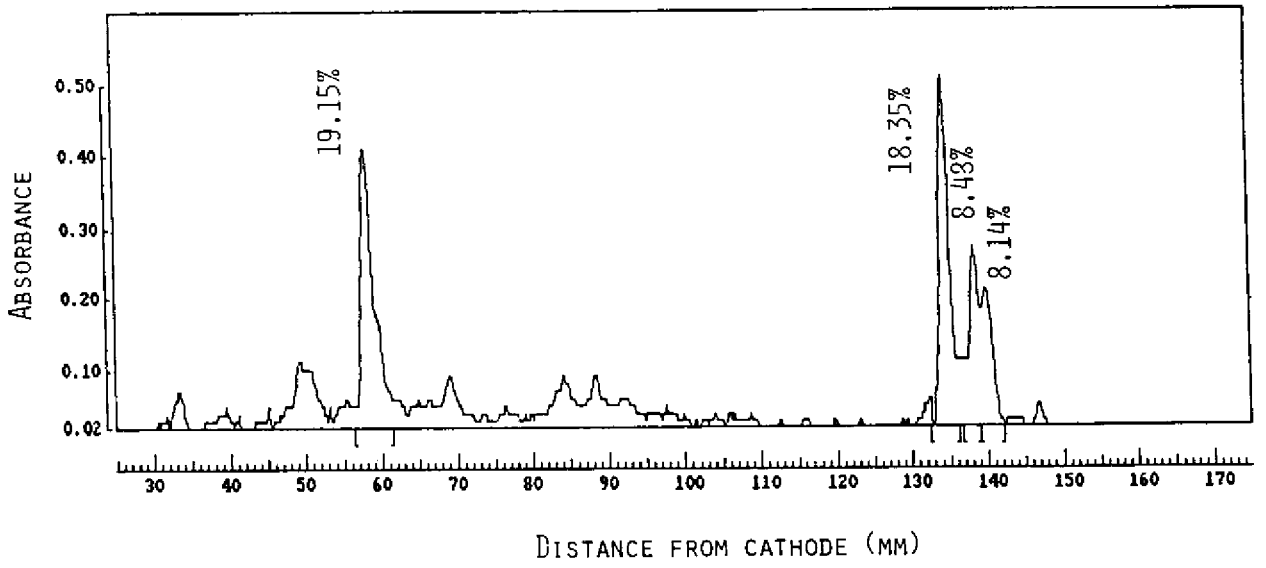

Figure 4. Densitometric profile of the water extract of raw pink shrimp. The peak area percentage of the four major protein bands are 19.15% for band pI 6.81, 18.15% for pI 5.52, 8.48% for pI 5.46 and 8.14% for pI 5.42.

Blind study

Using the methods developed in this study, the percent contents by weight of shrimp in shrimp-surimi mixtures were determined. In test 1, the percent content of shrimp was determined to be 15.0 and 13.6, respectively, when the two surimi-specific bands, pI 7.17 and 7.11, were used respectively for calculation (Table 1). When the data related to the two shrimp-specific bands (pI 5.52 and 5.46) were used, the content was determined to be 9.0 and 7.6%, respectively. The average of these four readings for shrimp content was 11.3; the actual content in this unknown mixture was 10%. Similarly, the percent contents of shrimp in tests 2 and 3 were determined to be 12.6 and 5.8, respectively, when this approach was used. The actual shrimp content in these two unknown samples was 12.4 and 5.7%, respectively.

CONCLUSION

A method was thus developed to quantitate the percent content by weight of shrimp in surimi-shrimp mixtures using the isoelectric focused protein profiles of surimi, pink shrimp, and surimi-shrimp mixtures containing known percents of shrimp. When the peak area readings of two shrimp-specific (pI 5.46 and 5.52) and two surimi-specific bands (pI 7.11 and 7.17) were correlated with the protein

Table 1. Determination of the percent shrimp content by weight in unknown surimi-shrimp mixtures in blind studies using data related to surimi- or shrimp-specific bands for calculation

	Surimi content (%)	Shrimp content (%)	Average shrimp content (%)	Actual shrimp content (%)
Test 1				
Surimi				
Band 1	85.0[a]	15.0[b]	11.3	10.0
Band 2	86.4	13.6.		
Shrimp				
Band 1	91.0[c]	9.0[d]		
Band 2	92.4	7.6		
Test 2				
Surimi				
Band 1	85.2	14.8	12.6	12.4
Band 2	88.3	11.7		
Shrimp				
Band 1	90.6	9.4		
Band 2	85.5	14.5		
Test 3				
Surimi				
Band 1	95.8	4.2	5.8	5.7
Band 2	89.6	10.4		
Shrimp				
Band 1	95.2	4.8		
Band 2	96.0	4.0		

[a]Percent surimi content by weight as determined using data related to surimi specific protein bands for calculation.

[b]Percent shrimp content calculated by deduction the surimi percent content from 100%.

[c]Percent surimi content calculated by deduction the shrimp percent content from 100%.

[d]Percent surimi content by weight as determined using data related to shrimp specific protein bands for calculation.

contents in these bands, a linear relationship was derived. Based on this linear relationship between peak area readings and protein contents of the specific bands, and the peak areas of those specific bands in unknown samples, the percent content of shrimp or surimi in an unknown mixture could be determined. This method could be used to enable accurate labelling of the content of a specific seafood component in fabricated surimi products.

ACKNOWLEDGMENTS

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MONITORING LACTIC ACID RESIDUALS IN NATURAL AND TREATED SHRIMP

L.A. Applewhite and W.S. Otwell
Department of Food Science and Human Nutrition
University of Florida
Gainesville, Florida 32611

INTRODUCTION

Lactic acid and sodium lactate are established food ingredients in numerous products. They are selected for use in seafood primarily for their preserving properties. Lactic acid has been found to be a good antimicrobial agent, to control bacterial growth, and to affect melanosis when used as a dip for shrimp. To date, it has been noted that treatments with lactic acid, with concentrations in the recommended range, does not contribute any significant effect on color, texture or taste of the seafood.

OBJECTIVE

The purpose of this study was to accurately measure natural and residual levels of lactic acid in shrimp. With increasing use of lactic acid and various lactates as a seafood preservative there is a need to distinguish treated product.

MATERIALS AND METHOD

Ecuador white shrimp were used for all analysis and were deheaded, peeled, butterflied and frozen prior to analysis. Two 100g batches of shrimp were analysed at each sampling. One 100g sample, the control, was soaked in distilled H_2O for 10 minutes and the other 100g sample was soaked in either a 0.5%, 1.0% or 1.5% lactic acid solution also for 10 minutes. Both samples were then drained for 1 minute. Five 10g subsamples were analysed from each batch. After weighing, the raw shrimp meat was homogenized in a 10% TCA trichloroacetic acid) solution to precipitate the proteins, then centrifuged at 2000 rpm for 20 minutes to recover a supernatant for analysis. In a test tube, 2.0 ml of glycine buffer, 4.0 ml of distilled H_2O and 0.1 ml lactate dehydrogenase were added to 10 mg NAD (nicotinamide adenine dinucleotide). The tubes were capped and inverted until the NAD was dissolved. A 2.8 ml aliquot of the

NAD solution was pipetted into blank and test vials, 0.2 ml of the 10% TCA solution was added to the blank cuvet and 0.2 ml of the sample supernatant was added to the test cuvets. All cuvets were incubated at 25°C for 30 minutes. The absorbance of the sample cuvets was read at 340 nm verses the blank as the reference. Concentrations of lactic acid in the shrimp were determined from a standard curve. Standards were prepared in 10% TCA and analysed as above. The levels of lactic acid found in the control shrimp were subtracted from those for the treated shrimp to obtain lactic acid residual levels.

The enzymatic method of analysis used was a modification of a kit (Procedure No. 726-UV/826-UV) obtained from Sigma Chemical Company. This method utilizes lactate dehydrogenase which catalyzes the following reversible reaction:

$$\text{pyruvate} + \text{NADH} \xleftrightarrow{\text{LD}} \text{lactate} + \text{NAD}$$

To measure lactate, the reaction is carried out from right to left with excess NAD. The increased absorbance at 340 nm due to NADH formation is a measure of lactate present. A typical lactate calibration curve is linear from 2 to 100 mmoles lactic acid/L (Fig. 1). The specificity of the lactate dehydrogenase reaction in terms of interferences by various hydroxyacids was determined by several investigators. The possible interfering substances were not present in significant concentrations or else yielded substrate turnover rates too slow to cover any significant interferences in this study.

RESULTS AND DISCUSSION

The first group of shrimp were soaked and analysed, 13.4 mg of lactic acid per 100g shrimp was found in the controls and 30.1 mg lactic acid per 100g shrimp found in the treated shrimp (shrimp soaked in 0.5% lactic acid). The remaining shrimp were stored on ice in the refrigerator then soaked and analysed 2 days later. It was found that the lactic acid level in both the controls and the treated samples had increased. Two days later the analysis was repeated and again the lactic acid levels for both the controls and treated samples increased again. This process was continued over a period of 15 days (Fig. 2). Another 15 day series was done with shrimp soaked in water and 0.5% lactic acid and a similar trend was seen (Fig. 3) the lactic acid levels increased with refrigerated

FIGURE 1

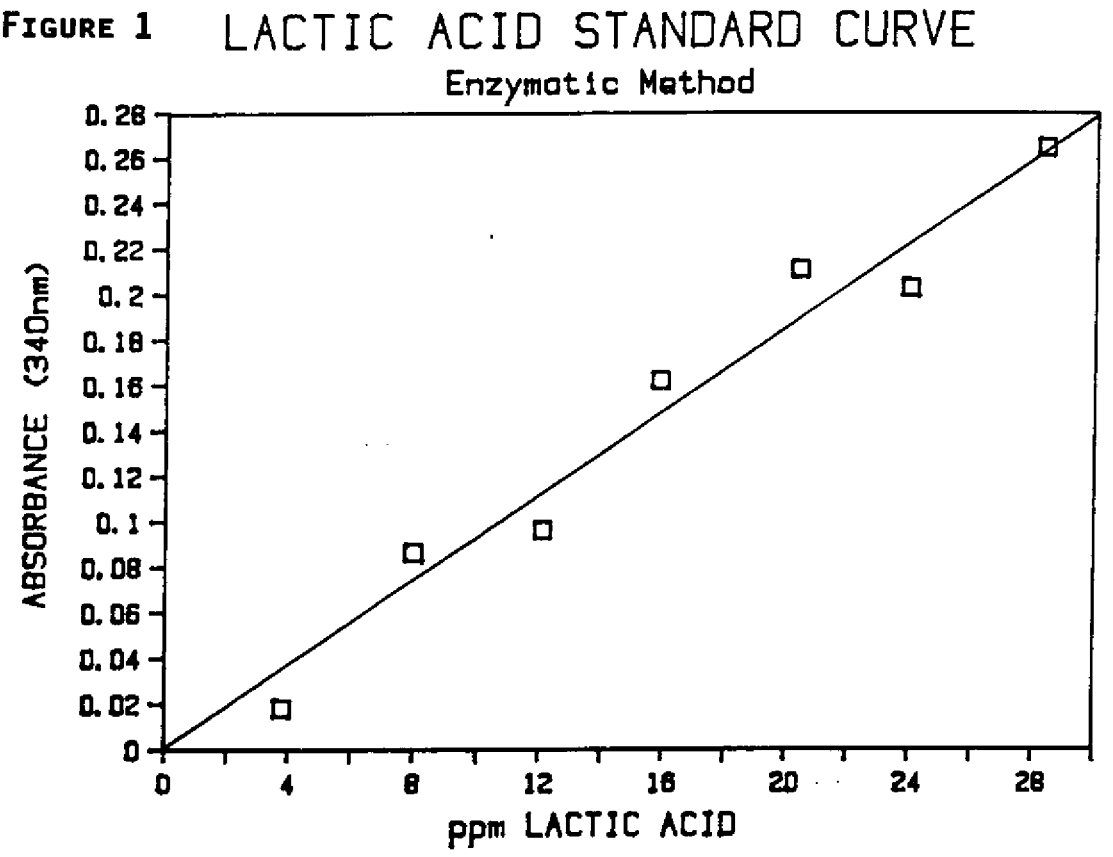

LACTIC ACID STANDARD CURVE
Enzymatic Method
ABSORBANCE (340nm)
0.28
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
4
8
12
16
20
24
28
ppm LACTIC ACID

FIGURE 2 LACTIC ACID ANALYSIS

(0.5% lactic acid soak 10 min)

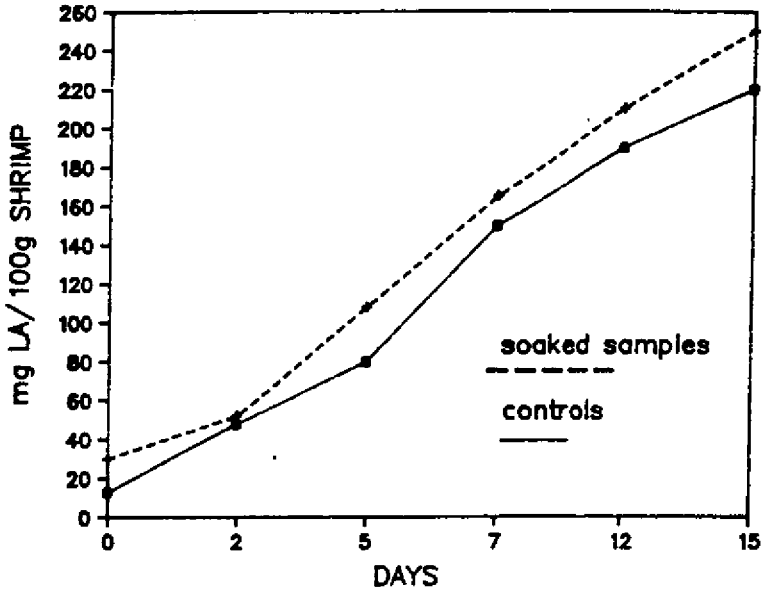
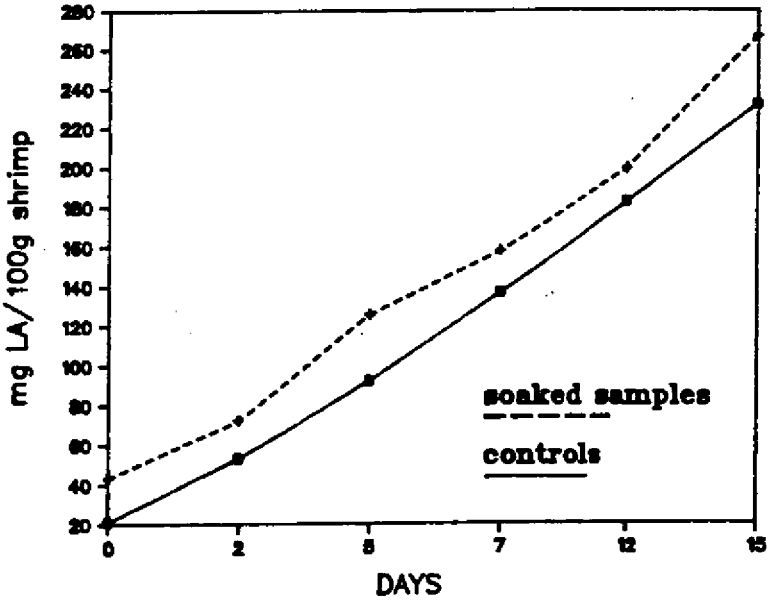


FIGURE 3 LACTIC ACID ANALYSIS

0.5% SOAK (2)



storage in both the control samples and the treated samples. The study was continued with shrimp soaked in 1.0% lactic acid and 1.5% lactic acid. Again, similar results were obtained (Fig. 4 and 5). Tables 1 through 4 show the reproducibility between the samples run at the various lactic acid contrentrations. Table 5 shows the means for all runs and the approximate difference in lactic acid concentration between the controls and the treated samples. It is interesting to note that the difference in lactic acid concentration between the controls and soaked samples was approximately 20mg/100g shrimp for both runs in which shrimp were soaked in a 0.5% lactic acid solution even though the initial (0 day samples) levels of lactic acid were different. As the concentration of lactic acid was increased the approximate difference between the controls and samples increased. The approximate difference between the samples soaked in a 1.0% lactic acid solution was 35mg lactic acid/100g shrimp and 50mg/100gram shrimp in the samples soaked in 1.5% lactic acid. It was also noted that for the soaks of higher concentrations of lactic acid (1.0% and 1.5%) that the residual levels of lactic acid after treatment were greater initially but not significantly greater after approximately 6 days.

FIGURE 4 LACTIC ACID ANALYSIS

(1.0% lactic acid soak 10 min)

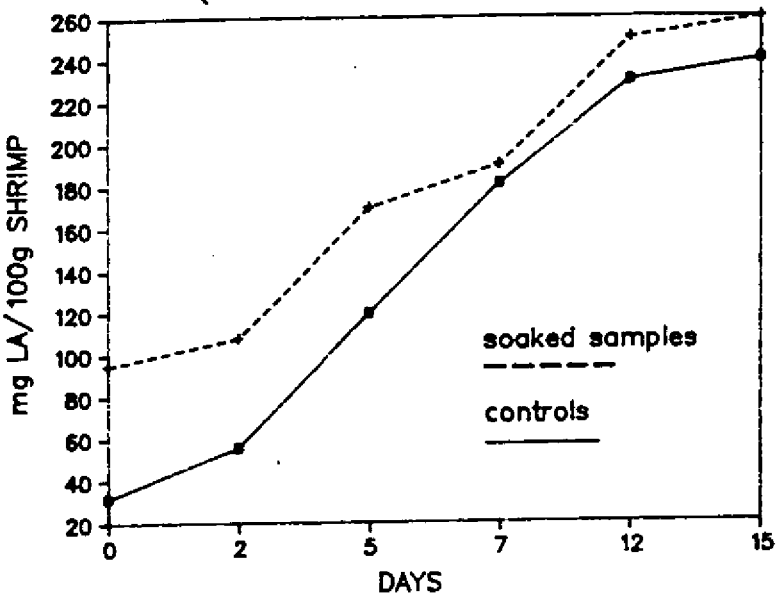


FIGURE 5 LACTIC ACID ANALYSIS

(1.5% lactic acid soak 10 min)

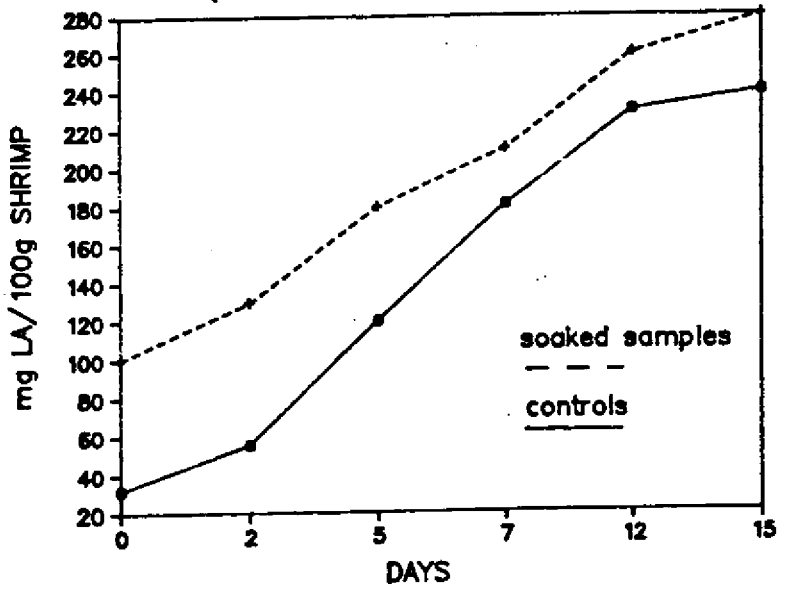


TABLE 1 LACTIC ACID ANALYSIS
0.5% SOAK

TRIAL 1

mg lactic acid/100g shrimp

CONTROLS

DAY	1	2	3	4	5	mean	RSD
0	13.4	13.2	11.4	12.9	13.4	13.0	3.2
2	46.8	48.0	48.0	50.0	48.8	48.3	2.4
5	78.2	81.4	79.8	80.2	80.8	80.1	1.5
7	147.9	150.2	149.2	152.6	151.1	150.2	1.2
12	190.1	188.4	189.6	192.9	190.4	190.3	0.8
15	220.4	218.6	217.9	222.2	221.8	220.2	0.7

SOAKED SAMPLES

DAY	1	2	3	4	5	mean	RSD
0	30.1	32.4	33.0	29.1	28.6	30.6	6.4
2	49.8	52.0	49.4	53.8	54.0	52.0	4.2
5	108.1	106.4	110.0	107.0	108.4	108.0	1.3
7	162.9	164.4	167.5	168.1	166.2	165.8	1.3
12	208.6	208.2	210.0	212.8	213.0	210.5	1.0
15	251.6	247.5	250.2	253.4	250.5	250.6	0.8

APPROXIMATE DIFFERENCE 20 mg LACTIC ACID/100g SHRIMP

TABLE 2 LACTIC ACID ANALYSIS
0.5% SOAK

TRIAL 2 mg lactic acid/100g shrimp

CONTROLS

DAY	1	2	3	4	5	mean	RSD
0	12.4	15.8	28.6	25.2	21.1	20.6	32.0
2	54.1	48.6	60.1	55.2	53.6	54.3	7.6
5	86.4	88.9	98.6	94.4	92.5	92.0	5.2
7	135.4	132.1	144.2	135.6	136.7	136.8	3.3
12	180.2	184.7	183.1	186.6	177.9	182.5	2.0
15	236.0	231.7	232.0	226.8	231.7	231.6	1.4

SOAKED SAMPLES

DAY	1	2	3	4	5	mean	RSD
0	43.1	40.6	40.4	46.5	46.1	43.3	6.7
2	71.1	72.6	73.6	69.9	75.0	72.4	3.0
5	125.2	126.1	122.2	130.0	126.4	126.0	2.2
7	149.3	154.8	158.0	167.7	162.4	158.4	4.4
12	199.0	198.5	195.3	203.4	200.3	199.3	1.5
15	262.1	268.7	271.2	266.8	265.4	266.8	1.3

APPROXIMATE DIFFERENCE 20 mg LACTIC ACID/ 100g SHRIMP

TABLE 3 **LACTIC ACID ANALYSIS**
1.0% SOAK

mg lactic acid/100g shrimp

CONTROLS

DAY	1	2	3	4	5	mean	RSD
0	38.4	38.2	40.1	44.9	40.4	40.4	7.0
2	60.4	66.1	59.4	58.6	60.2	60.9	5.1
5	120.6	118.2	122.0	124.5	124.3	122.0	2.2
7	160.3	158.7	165.5	160.2	159.1	160.8	1.7
12	227.8	230.1	235.2	227.4	230.4	230.4	1.5
15	249.6	255.3	246.7	250.6	250.1	250.5	1.2

SOAKED SAMPLES

DAY	1	2	3	4	5	mean	RSD
0	94.1	93.3	97.4	98.6	92.9	95.3	2.7
2	102.0	101.8	108.0	114.2	113.9	108.0	5.6
5	162.4	170.5	168.9	175.1	178.0	170.8	3.5
7	202.4	181.4	182.6	190.4	190.1	190.0	4.4
12	250.1	246.4	247.7	256.1	251.1	250.3	1.5
15	254.1	254.8	260.4	266.6	261.9	260.0	2.0

APPROXIMATE DIFFERENCE **35 mg LACTIC ACID/100g SHRIMP**

TABLE 4 LACTIC ACID ANALYSIS
1.5% SOAK

mg lactic acid/100g shrimp

CONTROLS

DAY	1	2	3	4	5	mean	RSD
0	28.8	29.1	36.4	24.9	36.8	31.2	16.0
2	55.1	50.1	56.6	62.0	57.8	56.3	8.0
5	120.5	118.1	115.5	128.0	117.4	120.0	4.1
7	176.3	181.0	186.6	189.0	177.8	182.1	3.0
12	228.7	232.1	227.9	233.4	230.4	230.5	1.0
15	236.1	237.8	244.4	243.1	241.2	240.5	1.5

SOAKED SAMPLES

DAY	1	2	3	4	5	mean	RSD
0	93.3	95.6	105.1	100.0	107.2	100.2	5.9
2	122.9	126.4	139.2	135.1	133.0	131.3	5.0
5	172.2	183.8	188.0	180.6	178.8	180.7	3.2
7	207.7	213.6	214.1	207.2	209.8	210.8	1.5
12	255.8	256.1	265.5	264.1	261.0	260.5	1.7
15	277.4	278.6	284.8	283.4	280.2	280.9	1.1

APPROXIMATE DIFFERENCE 50 mg LACTIC ACID/ 100g SHRIMP

TABLE 5 COMPARISON OF MEAN VALUES

	0.5% SOAK		1.0% SOAK		1.5% SOAK	
DAYS	CONTROL	SAMPLE	CONTROL	SAMPLE	CONTROL	SAMPLE
0	13.1	30.6	40.4	95.3	31.2	100.2
2	48.3	52.0	60.9	95.3	56.3	131.3
5	80.1	108.0	122.0	170.8	120.0	180.7
7	150.2	165.8	160.8	190.0	182.1	210.8
12	190.3	210.5	230.4	250.1	230.5	260.5
15	220.2	250.6	250.5	260.0	240.4	280.9

APPROXIMATE DIFFERENCES mg LACTIC ACID/100g SHRIMP

20 35 50

	0.5% SOAK	
DAYS	CONTROL	SAMPLE
0	20.6	43.3
2	54.3	72.4
5	92.0	126.0
7	136.8	158.4
12	182.5	199.3
15	231.6	266.8

APPROXIMATE DIFFERENCES mg LACTIC ACID/100g SHRIMP

20

CONCLUSIONS

It was concluded that the shelflife was extended in shrimp treated with 1.5% lactic acid solutions and less (1.0% and 0.5%) without any negative attributes to the quality of the shrimp. Also that it is difficult if not impossible at this point to determine if shrimp have been treated with lactic acid since the natural levels vary. Storage temperature and time also plays a significant role in lactic acid levels of both natural and treated shrimp.

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STUDIES ON BACTERIAL GROWTH AND HISTAMINE PRODUCTION ON VACUUM PACKAGED TUNA

C. I. Wei, C.-M. Chen, J. A. Koburger, W. S. Otwell and M.R. Marshall

Food Science and Human Nutrition Department, University of Florida
Gainesville, Florida 32611

INTRODUCTION

Scombroid poisoning is a foodborne intoxication caused by the consumption of scombroid fish containing hazardous levels of histamine in the muscle tissue (Arnold and Brown, 1978; Behling and Taylor, 1982). Histamine is produced by microbial decarboxylation of the free histidine in the tissue as a result of improper handling of the fish (Arnold and Brown, 1978; Eitenmiller et al., 1981). *Klebsiella pneumoniae* (Taylor et al., 1979), *Morganella morganii* (formerly *Proteus morganii*, Kawabata et al., 1956; Sakabe, 1973) and *Hafnia alvei* (Ferenick, 1970; Havelka, 1967) have been implicated as causative organisms in the formation of toxicologically significant levels of histamine in fish.

Low storage temperatures are used in the fishery industry to control bacterial histamine production. Recently, Arnold et al. (1980) showed that histamine production by *M. morganni* and *Morganella vulgaris* in tuna fish infusion broth (TFIB) was delayed and diminished at 7°C. A similar effect was noted for the slow histamine producer, *H. alvei*. Behling and Taylor (1982) showed the lower temperature limits for production of toxicologically significant levels of histamine in TFIB were 7°C for *K. pneumoniae*, 15°C for *M. morganii* 110SC2 and JM, and 30°C for *H. alvei*. *K. pneumoniae* virtually ceased to produce histamine at 0° or -3°C though the organisms still survived over an extended period of time. Recently, Chen et al. (1988) also showed the low temperature limit for *K. oxytoca*, *M. morganii*, and *H. alvei* for growth in TFIB was 5°C, 7°C and 3°C, and for histamine production was 7°C, 7°C and 20°C, respectively.

Vacuum packaging is used increasingly in the seafood industry to pack frozen seafoods to produce high quality and more acceptable products than presently being offered to the consumers (Anderson, 1983). Vacuum-packed seafoods usually have a longer shelf-life. Clingman and Hooper (1986) found that fresh fish products stored under vacuum packaging had an overall increase of 7 days over aerobically stored fish.

Since gas permeability of the plastic film can affect microbial growth, discoloration of the flesh, development of putrefactive odors and thus the shelf-life of the meat, similar results are expected to occur for vacuum-packaged seafood. Limited information is available regarding the growth of histamine-producing bacteria and the levels of histamine production in tuna samples subjected to vacuum packaging. Therefore, the use of vacuum packaging coupled with low temperature storage on the shelf-life of tuna was examined in this study. Niven's agar medium was used together with Bacto plate count agar to enumerate the time-related changes of histamine-producing bacteria and total aerobic counts, respectively.

MATERIALS AND METHODS

Preparation of bacterial suspension for spiking

Klebsiella oxytoca T_2 (formerly *K. pneumoniae* T_2), *Morganella morganii* JM (formerly *Proteus morganii* JM), and *Hafnia alvei* T_8 were provided by Dr. S. L. Taylor at the Department of Food Science and Technology, University of Nebraska. Bacteria maintained on trypticase soy-histidine (2%) agar slants were inoculated into trypticase soy broth-histidine medium (TSBH) and incubated at 28°C for 24 hr. Aliquots (0.2 mL) of these were then transferred into fresh TSBH and incubated for an additional 18 hr prior to use for spiking tuna samples.

Vacuum packaging material

Oxygen-barrier storage bags (25.4 x 9.0 cm) were provided by the Cryovac Packaging Corp. (Simpsonville, SC). The oxygen permeability rate of the bags at 73°F was 4,000 mL/m^2/24 hr, and the moisture transmission rate at 100°F was 0.65 g/100 in^2/24 hr at standard conditions of 100% relative humidity.

Bacterial spiking and vacuum packaging of tuna samples

Fresh yellowfin tuna (*Thunnus albacares*) loins of 15-20 kg each were obtained one-day after landing from a wholesale fish distributor in Tampa, FL and transported in ice to the Food Science and Human Nutrition Department at the University of Florida. The outer layers of the tuna loins were removed carefully with a sterile knife. Tuna samples of approximately 10x5x3 cm were randomly divided into two groups (non-vacuum vs vacuum packed), and then each into 4 subgroups: tuna only, and tuna spiked with *K. oxytoca* T_2, *M. morganii* JM or *H. alvei* T_8.

Bacterial suspensions for spiking were prepared by adding 0.5 mL of the individually activated culture in TSBH to 500 mL of sterile Butterfield's phosphate buffer (final concentration for *K. oxytoca* T_2: 5.6 x 10^6 cells/mL; *M. morganii* JM: 1.3 x 10^6/mL; and *H. alvei* T_8: 1.2 x 10^6/mL). After the fish samples were dipped into the bacterial suspensions for 20 sec and the extra liquid drained off, the samples were placed into the oxygen-barrier bags and subjected to vacuum packaging, or sealing with wires (for the non-vacuum packaged control). Vacuum packaging was done using Reiser vacuum-packaging equipment at a pressure of 1 bar for 15 sec: 10 sec for vacuum build-up and 5 sec for sealing. Both vacuum and non-vacuum packaged samples were stored at 2°C or 10°C for 15 days. At each time interval (0, 3, 6, 10 and 15 days), duplicate samples were randomly removed from each subgroup and processed for total aerobic counts, differential plate counts and histamine analyses.

Tuna sample processing for bacterial enumeration and histamine quantitation

At the end of each incubation period, a 25-g portion of the tuna samples were removed and homogenized with 225 mL 0.9% normal saline solution in a Waring Blender for 2 min at 4,000 rpm. After pH measurement, the homogenates were serially diluted with Butterfield's phosphate buffer and surface-plated on Bacto plate count agar (Difco Laboratories) and Niven's differential agar (Niven et

al., 1981; Chen et al., 1989). The plate count agar was incubated at 28° while Niven's agar was incubated at 35°C for 24 hr. Four plates were used for each dilution.

For histamine analysis, a 25-g portion of the tuna sample was homogenized with 30 mL 6% PCA in a Waring Blender for 2 min at 4,000 rpm. The homogenate was filtered by suction through a Whatman # 1 filter paper. The PCA extracts were brought to 100 mL with 6% PCA solution followed by titrating the extracts with a 30% KOH solution to adjust the pH to 7.0-7.3. After potassium perchlorate precipitate was removed, the final extracts were filtered through a 0.45 μm filter, the samples were subjected to HPLC analysis.

Histamine quantitation

Quantitation of histamine was accomplished using a modified ion-moderated partition HPLC method (Gill and Thompson, 1984; Chen et al., 1989). The set-up of the HPLC system and the operation conditions were described in a previous report (Chen et al., 1989). Histamine standard solutions (1, 2, 4, 8 and 10 mg%) were prepared from the 100 mg% stock solution by diluting with 6% PCA-30% KOH.

Duplicate test samples were used and each extract was analyzed at least twice. For some samples, further dilution of the final extracts was needed due to high histamine content. Final histamine concentrations were calculated according to the following formula:

$$C_2 = \frac{C_1 \times (V_1 + V_2) \times D}{V_3 \times 0.93}$$

where C_1 was the concentration of histamine derived from the standard curve (mg/mL); C_2 was the final concentration of histamine in tuna (mg/100 g tuna). V_1 was the volume of PCA extract neutralized (100 mL for tuna); V_2 was the amount of 30% KOH added; V_3 was the amount of tuna (25 g) used; 0.93 was the extraction recovery rate and D was the dilution factor.

RESULTS AND DISCUSSION

Since the low temperature limit for *K. oxytoca*, *M. morganii*, and *H. alvei* for growth in TFIB was 5°C, 7°C and 3°C, and for histamine production was 7°C, 7°C and 20°C, respectively, storage temperatures of 2°C and 10°C were used in this study.

Tuna samples showed a slight increase in pH values after storage for 15 days. Fresh tuna (day 0) homogenate in 0.9% saline had a pH of 6.0-6.2. Those stored for 15 days under vacuum packaging at 2°C and 10°C, and those under non-vacuum packaging at 2°C and 10°C were determined to be pH 6.2-6.6, pH 6.3-6.8, pH 6.0-6.6 and pH 6.4-6.8, respectively. Apparently, the increased bacterial growth at 10°C resulted in the pH changes, possibly due to the increased production of amines.

Bacteria grew rapidly on tuna samples stored at 10°C (Fig. 1). Vacuum packaging of tuna samples did not inhibit or slow bacterial growth. By day 6, the total aerobic counts in all the four subgroups in vacuum and non-vacuum

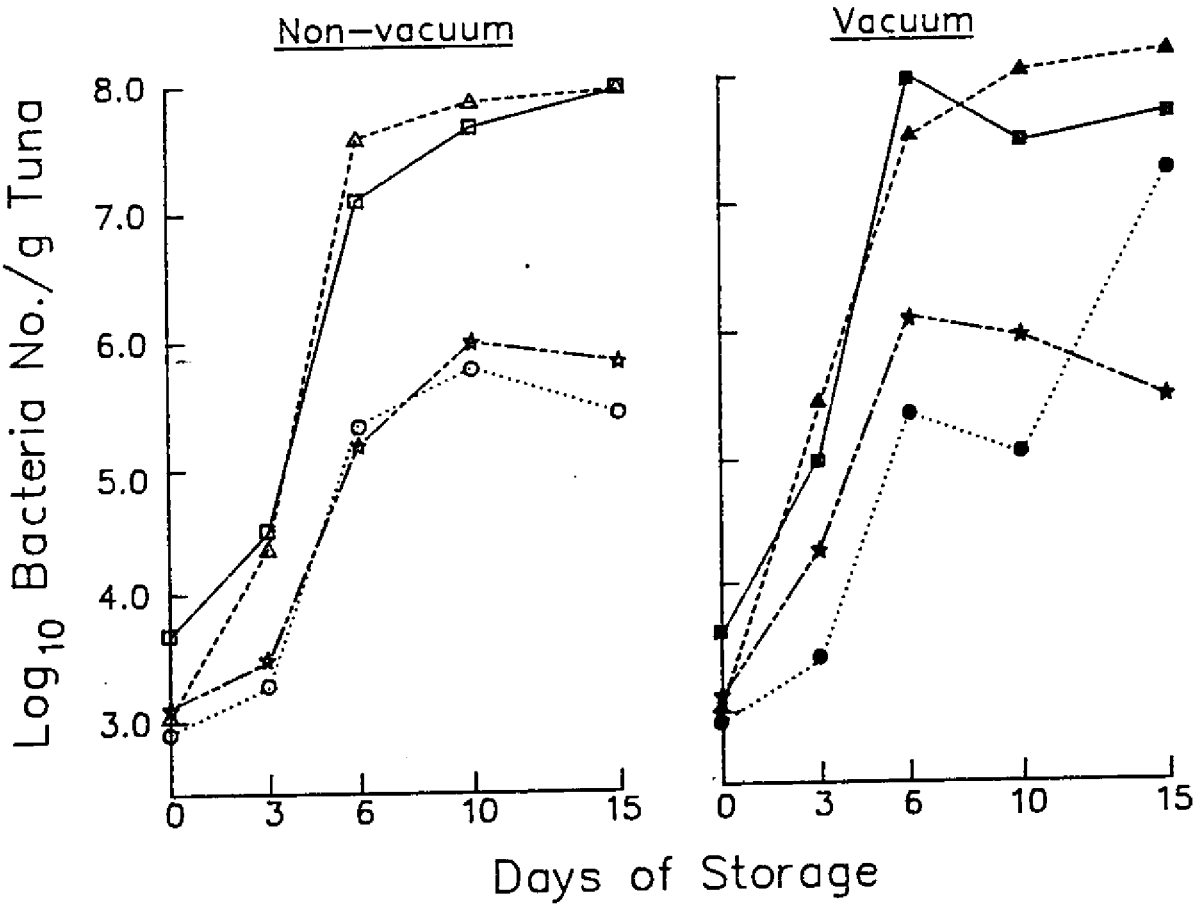

Figure 1. Time-related changes of bacterial growth at 10°C on non-vacuum andvacuum-packaged tuna samples as determined using Niven's agar. ○ , ● : tuna only; △ , ▲ : tuna spiked with *K. oxytoca*; □ , ■ : tuna spiked with *M. morganii*; and ☆ , ★ : tuna spiked with *H. alvei*.

packaged groups reached a level of 10^7 cells/g tuna (data not shown). All the tuna samples stored at this temperature started to produce putrefactive odors and surface slime by day 3. The samples also showed undesirable color changes.

The overall numbers of histamine positive colonies on Niven's agar at each time period were of the same order of magnitude as the total aerobic counts for tuna samples spiked with *Klebsiella* or *Morganella* and subjected to vacuum or non-vacuum packaging. The number of histamine producers also reached 10^7 cells/g tuna by day 6. Niven's agar was reported to detect with 95.8% and 93.9% accuracy the histamine producers from temperature-abused and bacteria-spiked tuna samples (Chen et al., 1989).

Samples spiked with *Hafnia* had fewer positive colonies on Niven's agar than on plate count agar; reaching only 5×10^5 colonies/g by day 15. Niven's agar has been reported not to be a satisfactory medium for *H. alvei* (Chen et al., 1989). This might have contributed to the significant difference between the differential counts and the total aerobic counts. The tuna control subgroups also had less differential counts than total aerobic counts, although the total aerobic counts of the subgroup subjected to vacuum packaging reached 10^7/g on day 15. The change of the environment to microaerophilic or anaerobic might enhance the growth of some histamine producers on the non-spiked tuna samples.

Tuna samples stored at 2°C showed increases in total aerobic counts although the extent of increase was not as dramatic as at 10°C (Fig. 2). These samples still developed odors but only after about 10 days of storage when the numbers of total aerobic counts were about 1 log higher than the initial counts (10^3 cells/g tuna, data not shown). Unlike the total aerobic counts, the numbers of differential colonies of these tuna samples on Niven's agar did not change significantly over 15 days (Fig. 2). These three histamine producers did not multiply at 2°C, and the increases of the total aerobic counts would be due to the growth of some psychrotrophic microorganisms.

Significant amounts of histamine were produced on tuna samples stored at 10°C (Fig. 3). Apparently, the rapid increase in bacterial numbers at this temperature contributed to the rapid increases of histamine. It was interesting to note that tuna samples subjected to vacuum packaging had higher histamine levels. Tuna samples spiked with histamine-producing bacteria also had higher histamine contents than non-spiked ones.

Those tuna samples stored at 2°C still produced histamine but at greatly reduced levels (Fig. 3). Again the vacuum packaged groups had higher histamine levels than the non-vacuum packaged ones.

The results thus clearly indicated that low storage temperature was more important than vacuum packaging in controlling histamine production on tuna samples. At both storage temperatures (2° and 10°C), the vacuum-packaged group in general had higher histamine level. Although Clingman and Hooper (1986) indicated that vacuum packaging prolonged the shelf-life of fresh fish by suppressing the growth of psychrotrophic aerobic organisms associated with spoilage, the results from this study did not show that, possibly because the spiked bacteria could still grow at low temperatures. Although *K. oxytoca*, *M. morganella* and *H. alvei* were determined in TFIB not to grow and produce histamine

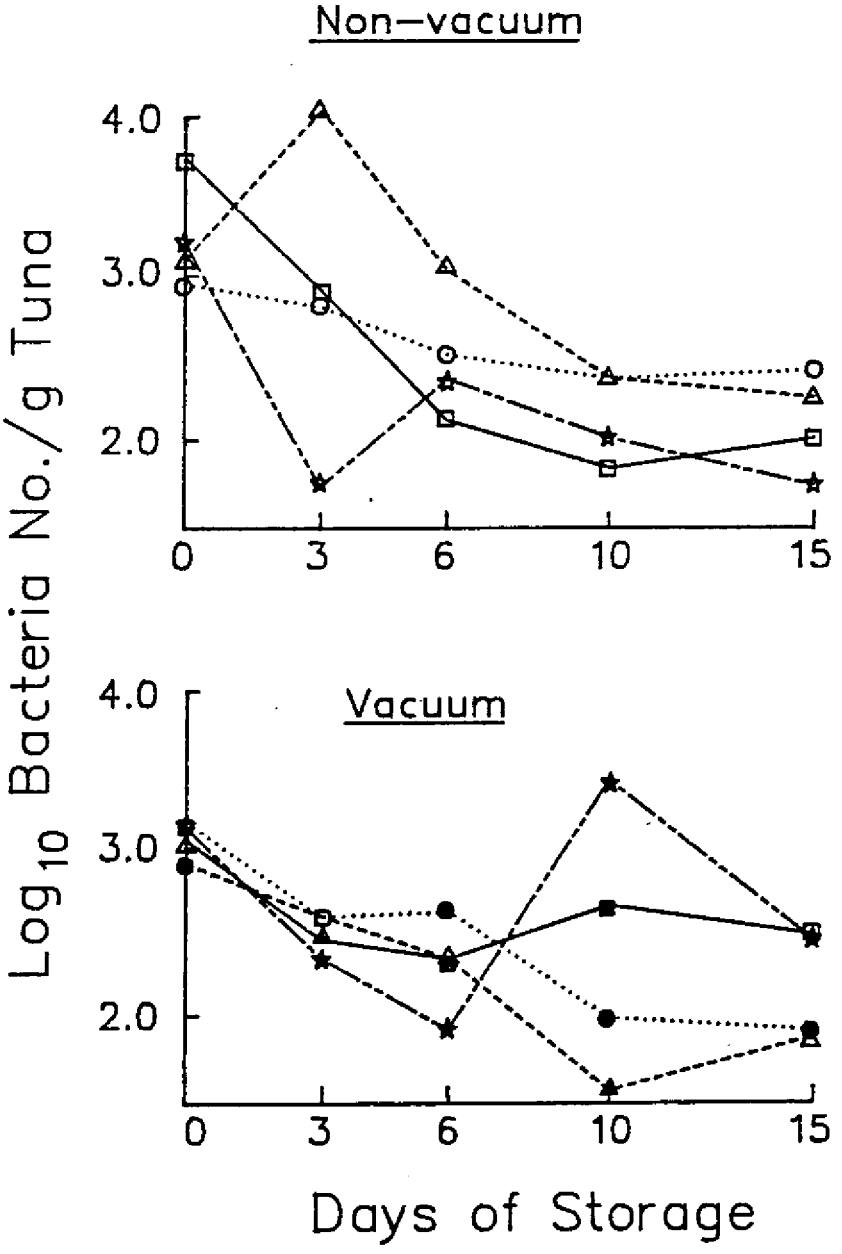

Figure 2. Time-related changes of bacterial growth at 2°C on non-vacuum and vacuum-packaged tuna samples as determined using Niven's agar. ○ , ● : tuna only; △ , ▲ : tuna spiked with *K. oxytoca*; □ , ■ : tuna spiked with *M. morganii*; and ☆ , ★ : tuna spiked with *H. alvei*.

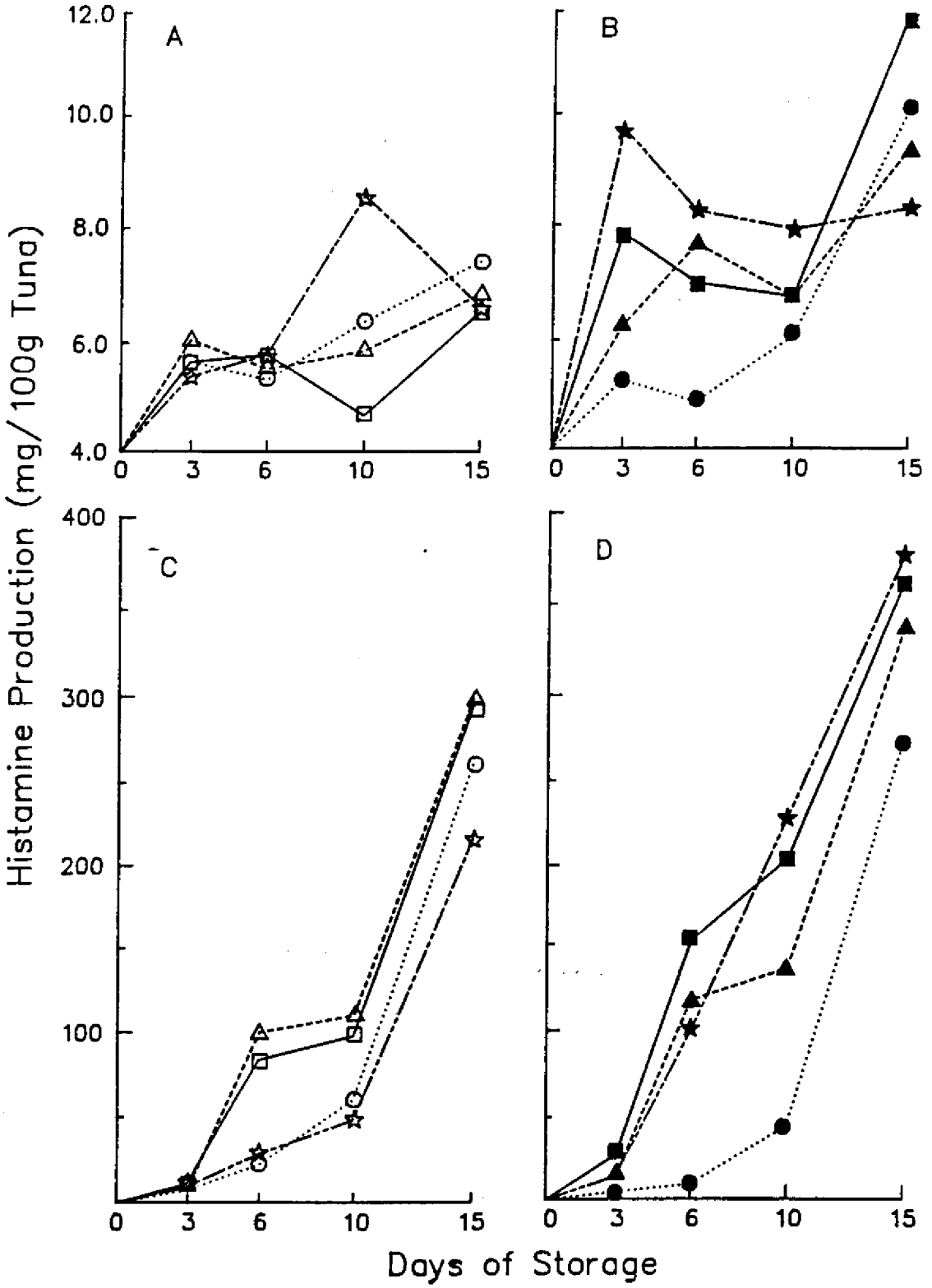

Figure 3. Time-related bacterial histamine production on tuna samples stored at 2°C (A and B) or 10°C (C and D). Groups B and D were vacuum packaged tuna samples, while A and C were non-vacuum packaged controls. ○ , ● : tuna only; △ , ▲ : tuna spiked with *K. oxytoca*; □ , ■ : tuna spiked with *M. morganii*; and ☆ , ★ : tuna spiked with *H. alvei*.

at temperatures below 3°C, these bacterial strains apparently still produced histamine on tuna samples stored at 2°C. The growth of other psychrotrophic microorganisms, as indicated by the slow increases of total aerobic counts at this temperature might contribute to histamine production. The results also indicated that only when the temperature throughout the distribution and storage of the product was carefully controlled (about 0°C), could the problem of microbial histamine production be inhibited and the shelf-life of the product extended.

ACKNOWLEDGEMENT

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CHILLPACK AND REFRIGERATED STORAGE OF POND-RAISED HYBRID STRIPED BASS FILLETS

L.C. Boyd, D.P Green, L.A. LePors

Department of Food Science, Box 7624
North Carolina State University
Raleigh, North Carolina 27695-7624

INTRODUCTION

With the current consumer demand to eat more fish and seafoods, many supermarket chains have added fresh seafood display counters whereas others have expanded their existing meat counters to accomodate seafoods. The general methods for displaying fresh fish are: (1) traditional storage on ice, (2) refrigerated storage at 2^{o} C (36^{o}F) and chillpack storage at -2^{o}C (28^{o}F). Though each of these methods has advantages and disadvantages, the need for extended storage stability and absence of fishy odors permeating the general food market are paramount to extending the availability of fresh seafoods to the consumer. However little information is available on the successful application of chillpack storage of warm water species such as farm raised hybrid striped bass.

This study was conducted to determine the effects of chillpack and refrigerated storage conditions on the chemical, physical and microbiological properties of hybrid striped bass and to compare these changes to sensory quality changes occurring during storage. Since product form will effect both product yield and nutrient composition (i.e. lipid content) the effects of product form on stoage stability was also determined

METHODS

SAMPLE PREPARATION: Fish used in this study were obtained from the Pamlico Aquacultural Research Center located at Aurora, NC. The fish were harvested, iced immediately and transported to Carolina Pride Seafood, Inc, Plylmouth, NC for processing. Approximately 120 fish weighing between 1.5 and 2.0 lbs. were commercially scaled, gutted, and rinsed. All fish were hand filleted with one-half of the fish having the belly flap left intack, whereas the second half was trimmed to remove rib bones and belly flap portion. The fish were further subdivided into two groups with treatment one designated as chillpack (CP) and treatment two designated as refrigerated pack (FR). Moisture proof, barrier-lined styrofoam trays were used to place fillets on prior packaging in oxygen permeable bags provided by Cryovac Division of W.R. Grace and Co, Duncan. S.C. A vacuum chamber was used to draw packages skin tight prior to closure.

Refrigerated packs were immediately placed in ice water, whereas chillpacks were sent through a liquid nitrogen tunnel (Liquid Air Product, Walnut Creek, CA) at appropiate time and speed to produce a surface chilled product. Refrigerated samples were stored at 2°C, whereas chillpack samples were stored at -2°C.

CHEMICAL AND MICROBIOLOGICAL EVALUATIONS:All samples were examined for physical, chemical, microbiological and sensory changes at day 0, 4, 8, 12, 15, 18, and 21 days of storage. Aerobic plate counts were performed in duplicate using trypticase soy agar with plates incubated at 25°C for 48 hrs (AOAC, 1984). Chemical analyses included proximate compostion, hypoxanthine (Hx) determinations, and 2-Thiobarbituric acid (TBA) number. Proximate composition analyses included moisture, protein, fat and ash of 100g compositie samples of raw and cooked samples according to standard AOAC procedures (1984). Hypoxanthine values were determined on 25g of minced tissue using the method of Woyewoda, et.al.(1986). This involved incubating neutralized extracts with xanthine oxidase at 37°C and measuring the absorbance at 290nm against a standard curve of uric acid. TBA values were determined on cooked homogenized fillets by the method of Salih et. al. (1980).

SENSORY EVALUATIONS: Samples were cooked to an internal temperature of 70°C in boiling water in specially designed pouches to allow the liquid to drain away from the sample during cooking. An 11 member trained panel provided quantitative descriptive analyses (QDA) of flavor, aroma and texture using an unstructured 15 cm line in which 0 represented the absence of a particular attribute and 15 the highest intensity rating. Prior to cooking the fish, an in-house and informal laboratory panel evaluated the raw packaged fish for spoilage using physical and sensory evaluatioin techniques involving odor, texture, color, and surface slime.
STATISTICAL ANALYSES: An analysis of variance and general linear model were used to analyze all data with mean differences submitted to Waller-Duncan test for significant differences using the Statistical Analysis System (SAS Institute, Cary NC). The experiment was performed on duplicate samples of fish obtained in May of 1989.

RESULTS

Table 1 shows yield data and proximate composition values for whole and trimmed fillets. The removal of the belly flap was reflected primarily through reductions in yield (i.e.,36.9 vs. 44.6) and increases in percent fat values of 4.52 over 2.54% for trimmed fillets.

TABLE 1: YIELD AND PROXIMATE COMPOSITION VALUES OF WHOLE AND TRIMMED FILLETS

SAMPLE	%YIELD	MOISTURE	FAT	PROTEIN
Headed/gutted	78.2	--	--	--
whole	44.6	73.2	4.52	19.52
trimmed	36.9	73.7	2.54	20.84

Analyses of microbiological , chemical and sensory data indicated that the refrigerated samples of both trimmed (FR-TM) and whole fillets (FR-BF) spoiled rapidly when compared to chillpack samples (i.e., CP-TM & CP-BF). Figure 1 indicates that the aerobic plate counts for refrigerated samples reached unacceptable spoilage levels of log 8.5/g in 8 days, whereas chillpack samples never exceeded log 6.6/g in 21 days of storage . No differences were observed between product form as whole and trimmed aerobic plate count values were similar to each other under both storage conditions. Physical and sensory evaluation of packaged raw fish prior to cooking indicated that spoilage was accompanied by bad odor, flesh gaping, and softness but very little slime formation.

FIGURE 1:TOTAL AEROBIC PLATE COUNT

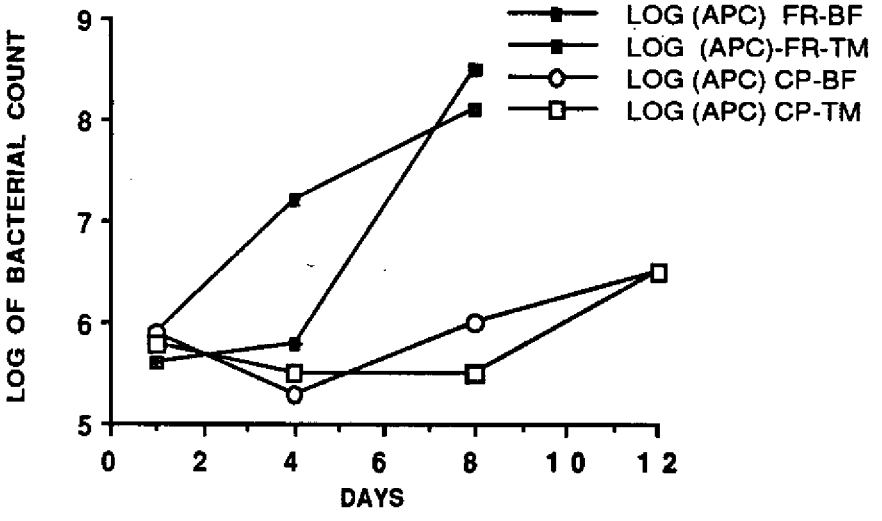


Figure 2 indicates that hypoxanthine values tended to follow microbiological data in that refrigerated samples also peaked at 8 days with values of 25 ug/100g of tissue compared to 7.6 ug/100g for chillpack samples. There was no difference in product form as Hx values for timmed and whole fillets were similar to each other for each storage treatment.
Figure 3 shows that mean TBA values of cooked samples increased slowly over the 21 day period , thus indicating that oxidative rancidity was occurring but very slowly. TBA values were observed to increase in all samples under both storage conditions for both TM and BF samples. However, no significant differences ($p<.05$) were observed between trimmed and whole fillets with belly flaps nor between storage treatments.

FIGURE 2: Hx VALUES OF FR vs CP SAMPLES

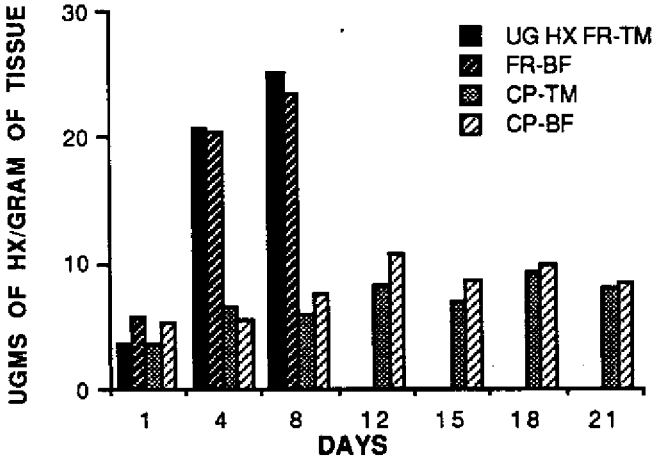
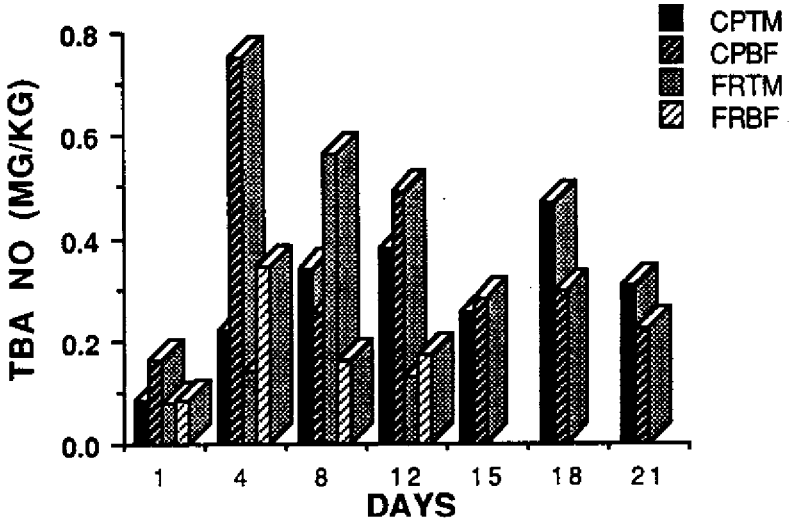


FIGURE 3: MEAN TBA NUMBERS FR vs CP SAMPLES



The sumary of mean panel intensity scores for aroma, flavor, and texture attributes is shown in table 2. No significant differences were observed between product form. However, several intensity notes were observed to have increased with storage. Mean panel scores for fishy flavor and oxidized flavor were observed to have increased with storage for both refrigerated and chillpack samples. No changes were observed for earthy, sour and fishy flavor notes. Both hardness and chewiness values for FR samples were significantly lower than CP samples. Panel scores for hardness and chewiness decreased in refrigerated samples, whereas the same attributes increased with increasing storage times in chillpack samples.

TABLE 2: SUMMARY OF MEAN SENSORY PANEL SCORES

TREATMENTS	HARDNESS	CHEWINESS	FISHY	OXIDATION
CP-BF	5.02^{A}	5.30^{A}	4.54^{A}	4.00^{A}
CP-TM	5.20^{A}	5.22^{A}	4.42^{A}	3.53^{A}
FR-BF	3.58^{B}	3.74^{B}	4.10^{A}	3.66^{A}
FR-TM	3.54^{B}	3.63^{B}	4.44^{A}	4.13A

Numbers followed by the same letter were not significantly different at $p<0.05$.

DISCUSSION

The shelf-life of commerically processed pond-raised hybrid striped bass was compared using storage temperatures of $2^{o}C$ and $-2^{o}C$ to simulate refrigerated and chillpack storage of fish, respectively. Results of the study indicated tha chillpack samples extended the shelf-life of samples by 13 days when compared to traditional storage without ice. These findings are significant in that many retail markets desiring to supply consumers with high quality seafoods might want to consider the extended storage stability and higher quality that can be obtained using chillpack storage conditions. Microbiological and hypoxanthine values were good indicators of product quality, whereas TBA values of lipid oxidation were not.

Since previous use of Hx values have shown that they are species specific, establishment of Hx values for a warm water species, such as striped bass are needed to give quick indicators of raw seafood quality over traditional use of APC numbers and trimethylamine values. The commercial significance of these findings depend upon supply beyond fresh marke demands. At the present time, premium prices are being paid to producers for whole fish of select size and weight. Once production exceeds the ability for absorbing the fresh supply or the prices to producers decline, the the opportunity for extended refrigerated storage of hybrid striped bass will be economically feasable. The inability of panelist to differentiate between fillets containing the belly flap portion and trimmed fillets is also of significance in that processors desiring to market the untrimmed fillet could expect to obtain higher yields without a lost in quality.

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VIBRIO VULNIFICUS IN RAW GULF COAST OYSTERS

Angela D. Ruple, David W. Cook and Rachel Dusho

Microbiology Section
Gulf Coast Research Laboratory
Ocean Springs, Mississippi 39564

INTRODUCTION

Vibrio vulnificus is a halophilic bacterium which is ubiquitous in warm estuarine waters. Oysters and other shellfish growing in estuarine waters can serve as a vehicle for transmitting the organism from water to man. Once transmitted to man, this organism has been identified as the causative agent of a primary septicemia that is often fatal. Because V. vulnificus represents a significant health hazard to a relatively small number of individuals with liver disease or compromised immune systems (5), but a much less significant risk to the population in general, it is difficult to establish regulations concerning its presence in shellfish. However, the presence of V. vulnificus in raw Gulf Coast oysters has become a concern for the industry as well as regulatory agencies.

In an effort to gain information about V. vulnificus that may be useful to both the shellfish industry and regulatory agencies, the Gulf Coast Research Laboratory began a study in January of 1989 to determine the levels of naturally occurring V. vulnificus in shellstock oysters arriving at Mississippi processing plants. Since the majority of the reported cases of primary septicemia associated with the consumption of raw oysters tend to cluster during the warm months of the year, a primary objective of this study was to determine if levels of naturally occurring V. vulnificus found in oysters arriving at processing plants show a seasonal variation.

In most of the reported cases of V. vulnificus associated primary septicemias, raw oysters from the shell rather than commercially processed oysters were implicated. This may be attributed to the observation that most of the commercially processed oysters are cooked before eating. However, oysters shucked in processing plants are frequently served raw in restaurants in the form of oyster cocktails or are placed on empty halfshells before serving. This may indicate that some step within normal commercial processing significantly reduces V. vulnificus levels in raw oysters. Therefore, steps in processing of oysters (shucking and washing) were investigated to determine their effects on V. vulnificus levels. Further, it is known that most vibrios do not survive well when stored in the cold, therefore, the effect of ice storage on the V. vulnificus levels in shucked oysters was also evaluated.

The information presented in this paper represents the first eight months of a one year study.

MATERIALS AND METHODS

Sample Collection and Handling

All of the oysters used in this study were Gulf Coast oysters harvested from approved areas outside Mississippi and shipped to Mississippi processing plants. For shellstock samples, 30 shell oysters from a single tagged sack were set aside for shucking and analysis in the laboratory. This number provided sufficient quantity of shucked meats to analyze two-200g samples from each lot of oysters. For processing studies, shellstock were collected as described above and the remainder of the oysters in the sack were shucked by plant personnel, washed on a skimmer according to normal plant procedures and packed into six 12 oz containers. Two of these containers were held at <10°C and analyzed within 2 hours of collection. Four additional containers were packed in crushed ice and analyzed after 3 and 7 days of storage.

Sample Preparation

Shellstock oysters were cleaned by brushing under running tap water and shucked with a sterile knife. Each sample consisted of 12-15 oyster meats homogenized in phosphate buffered saline PBS (8) for 1 minute. Decimal dilutions of 10^{-1} to 10^{-5} were prepared in PBS following the procedure of Cook and Pabst (6). All bacteriological media were inoculated from the appropriate dilutions. These dilutions allowed for determining *Vibrio* counts up to 110,000/g. Unless otherwise indicated all bacteriological media were Difco brand.

Standard Plate Count and Indicator Bacteria Analyses

Standard plate count and fecal coliform analyses were done according to recommended procedures (1) with the exception of using PBS as the diluent. The Most Probable Number (MPN) technique using lauryl tryptose broth followed by confirmation in EC broth was used to enumerate fecal coliforms. All EC broth tubes showing positive fecal coliforms were transferred to EC broth with MUG (10) for enumeration of *Escherichia coli*.

"Total vibrio" and Vibrio vulnificus Counts

A 3-tube MPN procedure using alkaline peptone water as the enrichment media was used to enumerate vibrios. One loopful of enrichment broth from tubes showing positive growth was streaked onto one plate each of TCBS and CPC (9) agars. All isolates that grew on TCBS were considered to be "vibrio-like" organisms and were used to calculate the total vibrio MPN.

Methodology for identification of V. vulnificus was that of Kaysner and Tamplin (9). TCBS and CPC plates were streaked from alkaline peptone enrichment tubes as described above. When typical colonies from the same enrichment tube were present on both CPC and TCBS plates, only those from the CPC plates were subjected to further testing. If these isolates could not be identified as V. vulnificus, those from the corresponding TCBS plate were tested. Typical colonies from CPC and/or TCBS were streaked for isolation on L-agar (10g tryptone, 10g NaCl, 5g yeast extract, 1.5% agar, 1L water, pH 7.4). Isolated colonies were then put on Motility Test Medium and Tryptic Soy Agar slants with 1% lactose added. Growth from the TSA-L slants was used to test for B-D-galactosidase (11), cytochrome oxidase (11), and slide agglutination with species specific H-antiserum (14). Isolates showing typical reactions from these tests were then inoculated into T_1N_1 broth (1% tryptone, 1% NaCl) and grown overnight at 35°C. One drop of the T_1N_1 broth culture was then used to inoculate the following biochemical media: Decarboxylase Basal Medium containing arginine, lysine or ornithine (8); tryptone broth with 0%, 3%, 6%, 8%, or 10% NaCl; Purple Broth Base containing 1% sucrose, lactose, mannitol, mannose, arabinose, salicin, cellobiose, maltose, trehalose, or galactose; and Hugh-Leifson Glucose Broth (8). All isolates showing typical biochemical reactions were used to determine the MPN per gram of V. vulnificus in the oysters.

RESULTS AND DISCUSSION

Shellstock

All of the shellstock were harvested from approved shellfishing areas and could be assumed to meet with National Shellfish Sanitation Program standards for shellstock oysters. However, previous studies (2,3,7,12,13) have shown that fecal coliforms can multiply in shellstock oysters during commercial harvesting and transport especially if oysters are held at temperatures above 10°C. Cook and Ruple (7) have shown that increases in both fecal coliform and vibrio levels appear to be governed by such factors as harvest and transport temperatures, and salinities. In order to gain some insight as to the handling of the oysters during transport, the overall quality of the oyster as measured by standard plate count and fecal coliform analyses was determined.

Standard plate count data is presented in Figure 1. Bars between the 1 and 2 represent January samples, bars between 2 and 3 represent February samples and so on. From January through August there was a gradual increase in the standard plate counts. However, even during the warm summer months, the standard plate count of the oysters arriving at the processing plants rarely exceeded the 500,000/g NSSP guideline (4).

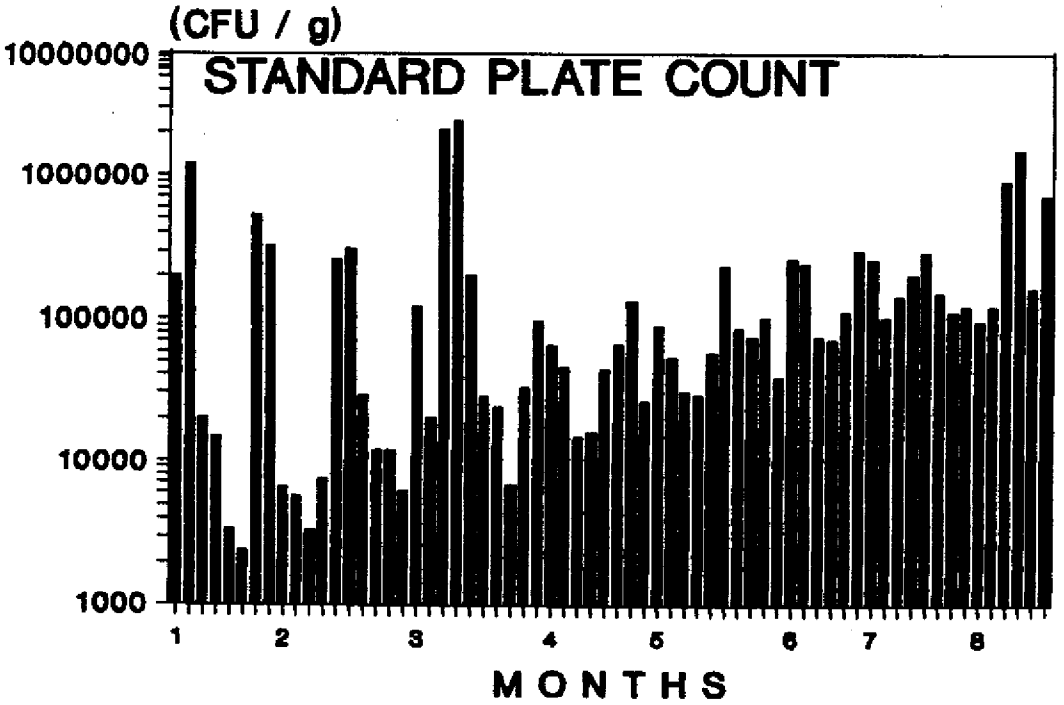
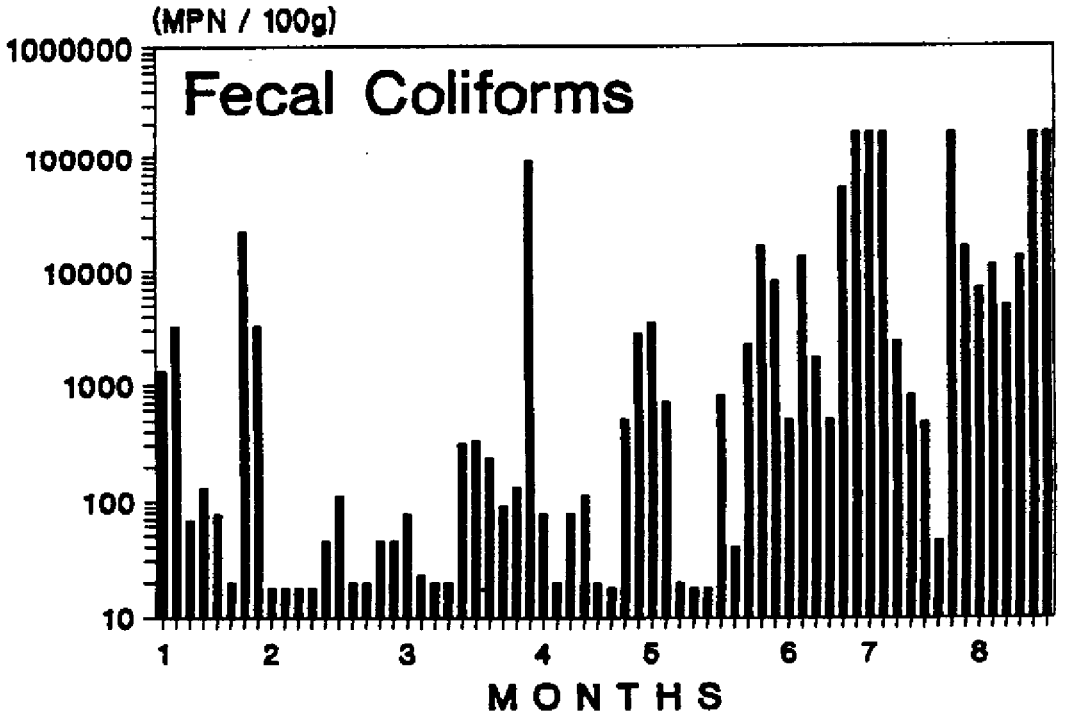


Figure 1. Standard plate counts of meats from shellstock oysters when received at processing plants.

During the early months of the study, fecal coliform levels (Figure 2) rarely exceeded 230/100g and were often below the minimal detectable level of 18/100g. During the warmer months of the study, fecal coliform counts were considerably higher and often exceeded the 230/100g guideline. Escherichia coli levels however generally remained low throughout the study period and rarely exceeded 230/100g. These increases in standard plate count and fecal coliform levels during the later months of the study were probably a response to increasing temperatures in the harvest areas.

Numbers of total vibrios (Figure 3) present in the shellstock oysters arriving at the processing plants showed much variation with levels in excess of 110,000/g often noted from April to August. During the early months of the study levels of V. vulnificus accounted for only a small portion of the total vibrios and were often below the minimal detectable level of 3 per gram. From April through August, V. vulnificus levels frequently equaled or exceeded 110,000/g.



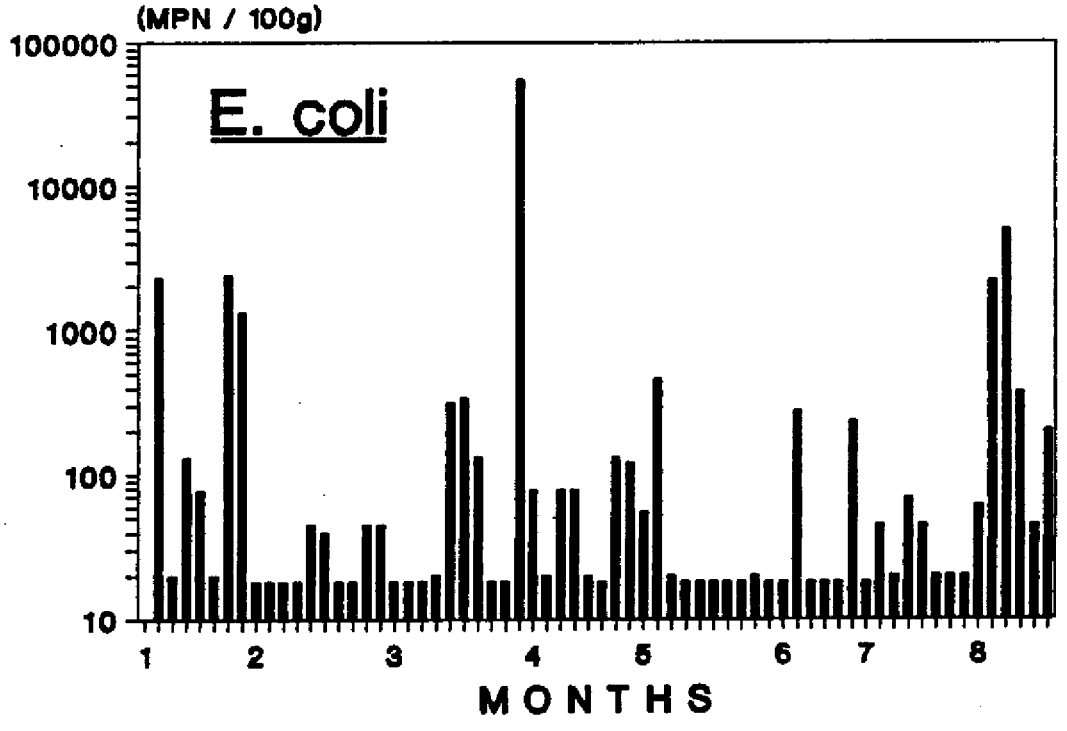
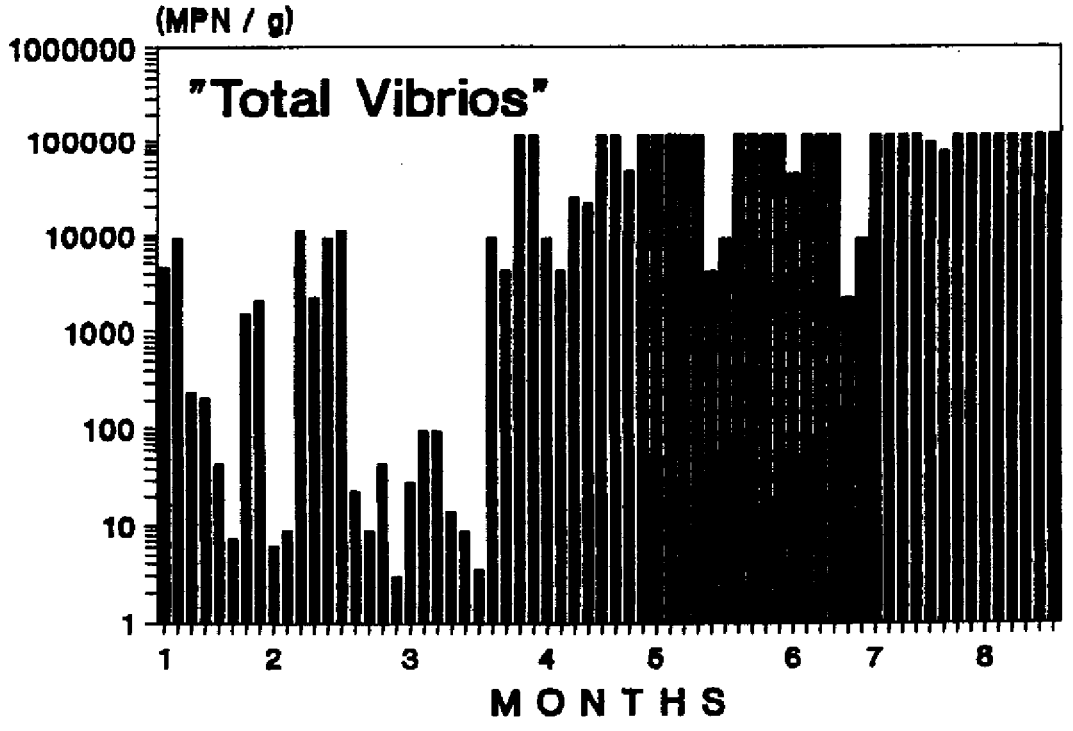


Figure 2. Indicator bacteria levels in meats from shellstock oysters when received at processing plants.



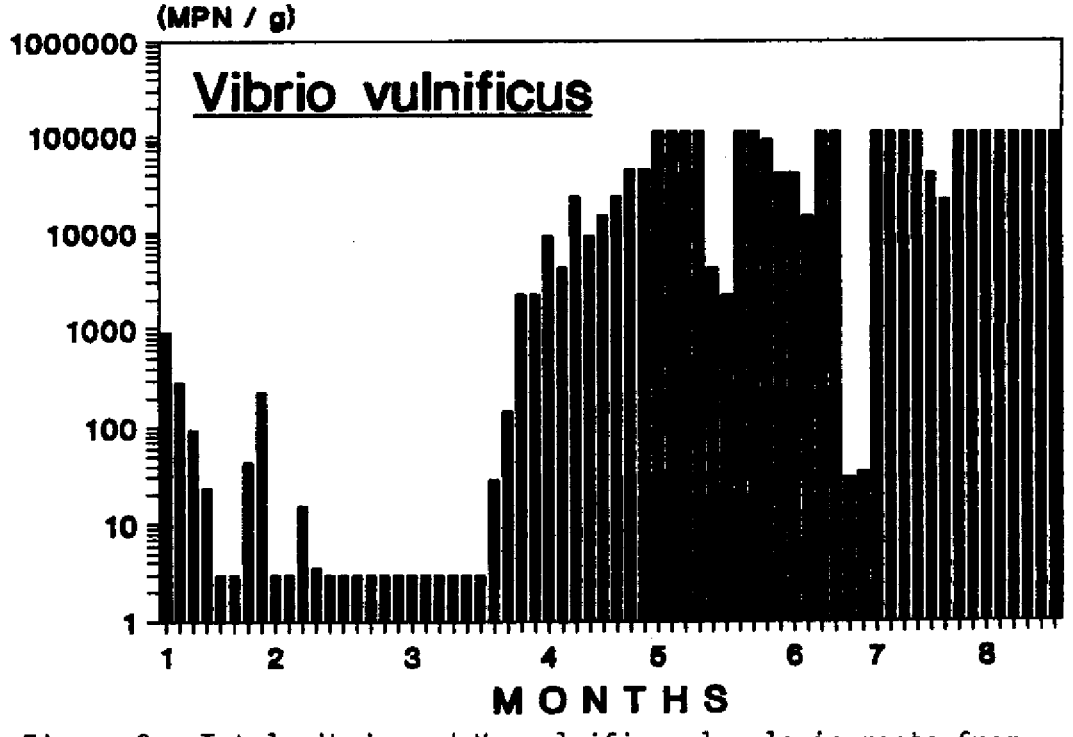


Figure 3. Total vibrio and V. vulnificus levels in meats from shellstock oysters when received at processing plants.

Table 1 summarizes the bacteriological data collected from shellstock oysters arriving at Mississippi processing plants during both the cool (January through March) and the warm (April through August) portion of the study period. Of the five bacteriological parameters measured, only E. coli did not show an increase in median counts during the warm period. The difference in the total vibrios and V. vulnificus levels was significant between the two periods.

Table 1. Bacteriological data obtained from shellstock oyster samples collected at oyster shucking plants. Range and median count data is given for two time periods.

	Oyster Samples Collected During	
	January - March	April - August
No. Samples	28	38
Std. Plate Ct. (CFU/g)	2,500 - 2,400,000[a] [26,000][b]	15,000 - 1,500,000 [100,000]
Fecal Coliforms (MPN/100g)	<18 - 92,000 [73]	<18 - >160,000 [2,300]
E. coli (MPN/100g)	<18 - 54,000 [20]	<18 - 4,900 [20]
Total vibrios (MPN/g)	3 - 110,000 [150]	2,300 - >110,000 [>110,000]
V. vulnificus (MPN/g)	<3 - 2,300 [<3]	<30 - >110,000 [110,000]

[a] - Range
[b] - Median

Processing Study

Shucking and washing of oysters according to normal processing procedures did not reduce the counts of naturally occurring V. vulnificus (Table 2). However, storage of processed oysters on ice for 3 days usually reduced these counts by greater than 90%. After 7 days of ice storage, the V. vulnificus counts were reduced to greater than 99% of the day 0 count. In the August storage study, both the shellstock meats and the day 0 processed meats had V. vulnificus counts in excess of 110,000/g. Therefore it was not possible to determine how great the reduction in counts were during storage.

Table 2. Effect of Processing and Ice Storage on V. vulnificus counts (CFU/g) in raw oysters. Each count represents the average from duplicate samples

Date Processed	Shellstock Meats	Processed Meats		
		Day 0	Day 3	Day 7
4/10/89	20,000	35,000	1,900 [94.6][a]	26 [99.9]
4/25/89	78,000	78,000	6,800 [91.3]	430 [99.5]
5/22/89	>110,000	>110,000	24,000 [=>78.2]	590 [=>99.5]
6/14/89	29,000	68,000	3,300 [95.2]	17 [99.9]
7/14/89	33,000	93,000	67,000 [39.1]	680 [99.4]
8/22/89	>110,000	>110,000	110,000	>57,000

[a] Percentage reduction during storage.

SUMMARY

Although this study has covered only eight months, seasonal variations in V. vulnificus levels in shellstock oysters were confirmed. These variations correspond to the cool and warm months when differences in incidence of V. vulnificus primary septicemia occur. Further, storage of processed oyster meats at low temperature reduces the levels of V. vulnificus and processed oyster meats are rarely implicated in cases of primary septicemia. These observations suggest that cases of V. vulnificus primary septicemia may be dose related. Further research in this area is suggested.

ACKNOWLEDGEMENT

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LOW DOSE GAMMA IRRADIATION OF VIBRIO VULNIFICUS IN BLUE CRAB (CALLINECTES SAPIDUS)

Robert M. Grodner
Department of Food Science
Louisiana Agricultural Experiment Station
Louisiana State University Agricultural Center
113 Food Science Building, Baton Rouge, LA 70803

Michael A. Watson
USDA Southern Regional Research Center
1100 Robert E. Lee Boulevard
New Orleans, LA 70179

INTRODUCTION

In Louisiana, the crab industry is a significant part of the economy. It has been estimated that 25.4 million pounds of blue crab were caught in 1984 (Anonymous, 1985). Additionally, the blue crab (Callinectes sapidus) is commonly found in the coastal waters of the United States, in the Gulf of Mexico and the Atlantic Ocean and is the basis of a large seafood industry (Tagatz, 1968).

Vibrio vulnificus has been implicated as a pathogen commonly associated with certain seafoods predominantly during the warmer months of the year (Kelly, 1982). This microorganism has also been found to cause septicemia in humans who handle or ingest crabs (Blake et al., 1979). In a study to determine the level of bacteria and vibrios present in crabs collected from Galveston Bay, it was ascertained that Vibrio vulnificus was the second largest quantity of the Vibrio species present in the hemolymph of 7% of the blue crab sampled from Texas coast(Davis and Sizemore, 1982).

Since the presence of this pathogen in crab may potentially cause serious health problems in individuals consuming contaminated crabs, gamma radiation has been suggested as a possible method of inactivating this microorganism in various seafoods.

Therefore, the purpose of this study was to determine the effect of pasteurization levels of gamma irradiation on Vibrio vulnificus in blue crab and the effect of refrigerated storage on the survival of the organism in the crab.

MATERIALS AND METHODS

Organism: Vibrio vulnificus 1008H was obtained from the Department of Microbiology, Louisiana State University. The organism was maintained

at room temperature on tryptic soy agar slants and transferred to fresh media at weekly intervals.

Preparation of Crab Homogenate: Fresh crab was purchased from local seafood markets. The nonsterile crab homogenate was prepared by blending one part peeled crab with one part sterile saline (3% NaCl) in a Waring blendor to form a smooth paste. The sterile crab homogenate was prepared in the same manner, except that the crab was sterilized at 121°C for 15 min before blending with saline.

Preparation of Inoculum: Growth phase cells of Vibrio vulnificus 1008H were prepared by cultivating the organism through two transfers on tryptic soy agar slants with 3% NaCl incubated for 18 hrs at 37°C. A loopful (approximately 0.1ml) of the organism was placed into 30 ml of tryptic soy broth(TSB) with 3% NaCl, placed on a shaker at 125 rpm, and incubated for 2 hrs at 37°C. This incubated 30 ml TSB served as the working culture.

After incubation, an appropriate quantity of the seeded broth was placed into another flask containing 50 ml TSB until a reading of 0.1 absorbance (600 nm) was obtained on the Bausch & Lomb Spectronic 70 (Bausch & Lomb, Inc., Rochester, NY). The seeded TSB was further incubated at 37°C until a reading of 0.65 absorbance was observed, which was equal to approximately 1 x 10^8 CFU/ml. The Vibrio vulnificus cell suspension was then centrifuged at 5000 rpm for 10 min in a Sorvall Superspeed RC5-C (Dupont Company, Newton, CT). The supernatant was discarded and the bacteria were resuspended in 20 ml of Alkaline peptone water (BAM, 1984). This new cell suspension represented a population of 1 x 10^8 V. vulnificus/ml. A known quantity of this prepared inoculum was added to the crab homogenate to produce a final concentration of 1 x 10^6 V. vulnificus/g crab meat. The inoculated samples were then placed in 125 ml Nalgene bottles, packed in ice, and transferred to the Nuclear Science Center at Louisiana State University.

Irradiation of Samples: The Nalgene bottles containing the samples were placed in a water tight chamber which was filled with ice and sealed. The chamber was lowered into the pit to expose the samples to the cobalt-60 source which emmitted 40 Gy/min. The samples were exposed to 0.15, 0.25, and 0.35 kGy. The bottles were then removed, packed in ice and transferred back to the Department of Food Science laboratory for analysis and storage. Control samples were treated in the same manner, except the irradiation treatments were ommitted (Hinton, 1983).

Enumeration of Vibrio vulnificus: Samples were examined for the presence of V. vulnificus at 0, 7, 14, and 21 days of storage at 4°C. Vibrio vulnificus in the sterile and nonsterile homogenates was enumerated by the MPN technique (BAM, 1984). The MPN tubes which contained 10 ml of Alkaline peptone water were incubated at 35°C for 16 to 18 hrs before streaking on to thiosulfate-citrate-bile salts-sucrose plates(BAM, 1984). After incubating the plates at 35°C for 18 to 24

hrs, typical V. vulnificus were removed for further examination (Hinton, 1983). Differential biochemical testing as prescribed by Hollis et al. (1976) for essential characteristics necessary for species specific identification were also implemented.

Irradiation of V. vulnificus in sterile and nonsterile crab homogenates at 4°C were performed in triplicate. The number of V. vulnificus surviving was calculated by determining the average number of V. vulnificus recovered from three samples subjected to the same irradiation treatment and refrigeration at 4°C (Figure 1).

RESULTS AND DISCUSSION

Effect of Radiation: In the nonsterile crab homogenate, the V. vulnificus population was reduced from 1.7×10^6 CFU/g to 2.7×10^2 CFU/g with 0.15 kGy of gamma radiation (Figure 2). An additional 0.10 Gy (0.25 kGy) treatment of gamma radiation reduced the initial 1.7×10^6 population to 2.1×10^1 CFU/g. No V. vulnificus bacteria were recovered at the 0.35 kGy treatment.

In the sterile crab homogenate, the V. vulnificus population was reduced from 1.6×10^6 CFU/g to 1.4×10^1 CFU/g with 0.15 kGy of gamma radiation treatment (Figure 3). An additional 0.10 kGy (0.25 kGy) treatment reduced the bacterial load of V. vulnificus to 5.8×10^0 CFU/g. Sterile crab homogenate yielded no V. vulnificus at 0.35 kGy treatment.

Effect of Time: Vibrio vulnificus was observed to survive longer in nonsterile crab homogenate compared with the viability shown in the sterile crab homogenate. The population of V. vulnificus in the nonsterile unirradiated crab was noted to decrease from 1.7×10^6 CFU/g on Day 0 to 5.1×10^1 CFU/g on Day 14 (Figure 2). The nonsterile homogenate treated with 0.15 kGy reduced from 2.7×10^2 CFU/g on Day 0 to 8.0×10^0 CFU/g of V. vulnificus on Day 14. Only 2.1×10^1 bacteria were recovered from the nonsterile homogenate treated with 0.25 kGy on Day 0. Vibrio vulnificus was not recovered from this treatment throughout the remainder of the 21 day refrigeration period. The microorganism was not recovered at the 0.35 kGy treatment.

In the non-irradiated sterile crab homogenate stored at 4°C, V. vulnificus decreased from its initial population of 1.6×10^6 CFU/g Day 0 to 2.9×10^1 CFU/g on Day 7 (Figure 3). No other V. vulnificus were recovered throughout the remainder of the 21-day refrigeration period. On Day 0, the 0.15 kGy and 0.25 kGy irradiation treatments yielded 1.4×10^1 CFU/g and 5.8×10^0 CFU/g V. vulnificus organisms, respectively. Growth of this bacterium was not observed throughout the remainder of this study. Vibrio vulnificus was not observed in the sterile crab homogenate treated with 0.35 kGy of radiation.

Survival in Sterile and Nonsterile Homogenates: As indicated, survival of V. vulnificus was more pronounced in the nonsterile crab homogenate than was evident in the sterile crab homogenate.

Figure 1:

MATERIALS AND METHODS FLOW CHART

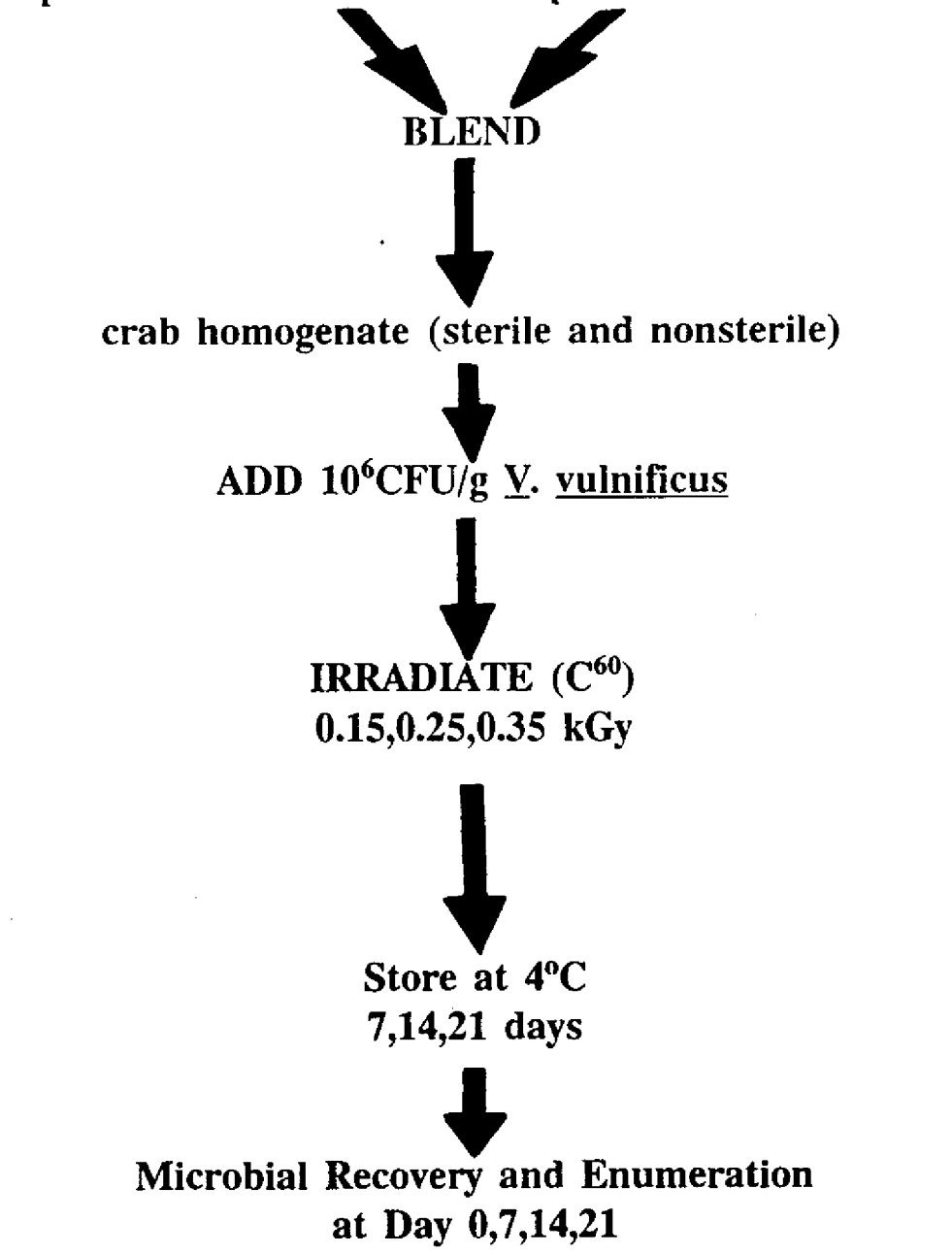


Figure 2: Effect of irradiation on Vibrio vulnificus in nonsterile crab homogenate stored at 4°C.

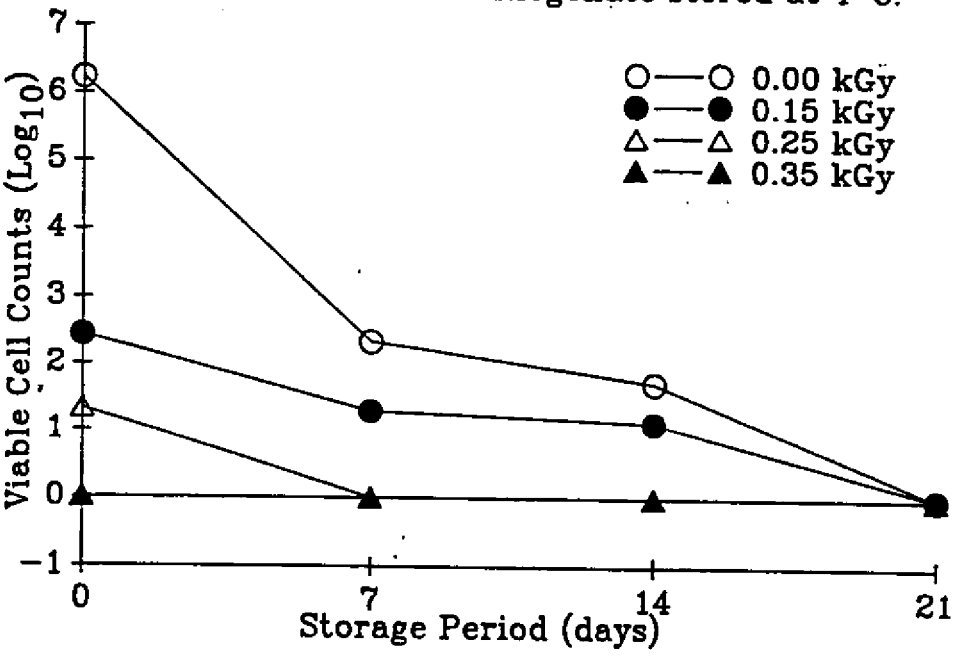
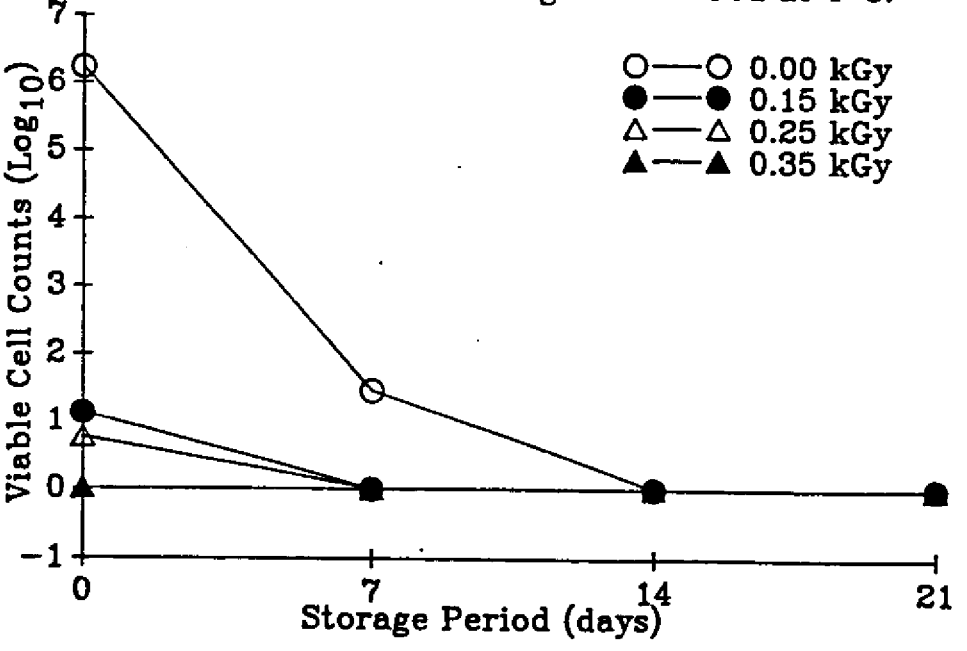


Figure 3: Effect of irradiation on Vibrio vulnificus in sterile crab homogenate stored at 4°C.



Previous research by Oliver (1981) indicated that V. vulnificus incurred a lower viability in oyster homogenate when refrigerated at 4°C within 24 hrs.

In the nonsterile crab homogenate, V. vulnificus was observed to survive up to 14 days at a refrigerated storage of 4°C. These data indicated that V. vulnificus may pose a potential health hazard to an individual consuming contaminated crabs.

The treatment of nonsterile crab homogenate with 0.15 kGy reduced the initial quantity of V. vulnificus of 2.7×10^2 CFU/g Day 0 to 8.0×10^0 on Day 14. Only 2.1×10^1 CFU/g V. vulnificus was recovered from the nonsterile homogenate treated with 0.25 kGy. No V. vulnificus was recovered from the nonsterile homogenate treated with 0.35 kGy.

In the sterile crab homogenate, the level of V. vulnificus was observed to be lowered by 5 log cycles. Additionally, both the 0.15 and 0.25 kGy treatments were noted to totally eradicate the presence of V. vulnificus on Day 7.

From the data presented, it was evident that the nonsterile crab homogenate was more conducive for the growth of this pathogenic microorganism than the sterile crab. One possible explanation may be that the microflora present in the nonsterile crab homogenate provided a more suitable pH essential for the normal growth of this bacterium. Another possible explanation may be that the V. vulnificus present in the sterile crab underwent enhanced sensitivity to the refrigeration temperature after the radiation treatment. Vibrio vulnificus has been reported to lose viability in oysters when stored at refrigeration temperatures (Oliver, 1981).

The use of gamma irradiation in this study has provided data to support the hypothesis that this form of processing is an excellent means of inactivating V. vulnificus from seafood.

SUMMARY AND CONCLUSIONS

The radiation resistance of Vibrio vulnificus in Louisiana blue crab was evaluated for 21-days at a refrigeration storage temperature of 4°C. Vibrio vulnificus was observed to survive from 7 to 14 days in both the sterile and nonsterile unirradiated blue crab. Results indicated that gamma radiation was quite effective in the inactivation of this estuarine pathogen. In particular, 0.35 kGy gamma irradiation was found to be immediately effective in the inactivation of 1.0×10^6 CFU/g of seafood from Day 0.

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Thermal Death Times for *Listeria monocytogenes* in Crabmeat

Mark A. Harrison* and Yao-wen Huang
The University of Georgia
Department of Food Science and Technology
Athens, Georgia 30602

INTRODUCTION

Listeria monocytogenes, a Gram positive bacterial foodborne pathogen, has been linked to foodborne illness outbreaks involving a variety of foods (1, 5, 8, 9). Listeric infections are particularly dangerous to pregnant women, infants, the elderly, and the immunocompromised with mortality rates in susceptible groups approximately 30% (7). *L. monocytogenes* is a psychrotrophic, facultative anaerobe which can grow in vacuum-packaged foods and can survive some degree of thermal processing (3, 6).

Much of the evaluation of the heat resistance of *L. monocytogenes* has been done using milk and pasteurization parameters. Less is known about the thermal resistance in muscle food. Farber (4) reported the decimal reduction times (D-values) of *L. monocytogenes* in ground meat ranging from 1.01 min at 62^{0} to 13.18 min at 56^{0}C.

Blue crab is a popular seafood item with more than 50% of the picked meat being pasteurized to offset seasonal fluctuations in some regions such as Georgia. Little information about growth and survival characteristics of *L. monocytogenes* in thermally processed refrigerated or frozen seafoods is known. Weagant et al. (12) surveyed frozen seafood products from several countries and found *L. monocytogenes* in 7 of 24 cooked crabmeat samples analyzed. The objective of this study was to determine the thermal death time for *L. monocytogenes* in blue crabmeat.

MATERIALS AND METHODS

L. monocytogenes (Scott A) was maintained on trypticase soy agar slants at 4^{0}C. Cultures were grown in trypticase soy broth for 24 h at 37^{0}C prior to inoculation into crabmeat.

Canned, pasteurized crabmeat was used which had a bacterial count of less than 10^{2}/g. The crabmeat was inoculated with approximately 10^{7} *L. monocytogenes*/g. A Kitchen Aid food processor (Model 4-c; Hobart Corp., Troy, OH) with the wire whip attachment was used to evenly mix the crabmeat and the *L. monocytogenes* inoculum. After 5 min of mixing, the inoculated crabmeat was transferred to a kitchen-type sausage stuffer and stuffed into collagen sausage casings (1.6 cm dia.; Teepak, Columbia, SC). Sausage casing was selected as the packaging material for individual crabmeat samples since small, uniform samples could be obtained for heating. In addition, the casing had minimal interference in the transfer of heat to the

product. Links (7.5 g) were formed and frozen at -10°C until used. Previous trials revealed little loss of viable L. monocytogenes due to freezing during a 3 wk storage period.

Prior to thermal processing the crabmeat portions were thawed at room temperature and one portion analyzed for the initial population of L. monocytogenes. A circulating water bath (Lauda K2/RD; Brinkmann, Westbury, NY) was used to heat the inoculated crabmeat to one of three temperatures, either 50, 55, or 60°C. For each thermal treatment, crabmeat portions were completely submerged below the water level in the preheated bath. One portion in each treatment contained a thermocouple connected to a multipoint recorder. The come-up time as well as the total time of processing at the desired temperature was recorded. After the desired temperature was reached, one portion was removed, placed on ice, and designated as time 0. Other portions were subsequently removed at designated times and also placed on ice.

The L. monocytogenes population of samples was determined by blending each in phosphate buffer (0.1 M, pH 7.0) and plating serial dilutions onto trypticase soy agar (Difco, Detroit, MI) and modified Vogel-Johnson agar (2) by the spread plate technique. The population of both uninjured and resuscitated L. monocytogenes was obtained by counting the colony forming units (cfu) that were present after the TSA plates were incubated for 48 h at 37°C. The population of uninjured L. monocytogenes was also determined by counting the cfu that were present after the modified Vogel-Johnson agar was incubated for 48-72 h at 37°C. Randomly selected colonies from each sample were screened for confirmation as L. monocytogenes as described previously (3).

Two replications of each heat treatment were done. D-values were calculated by the partial sterilization technique described by Toledo (10) using the following relationships:

$$D = \frac{\theta_2 - \theta_1}{\log N_1 - \log N_2} \qquad (1)$$

where $\log N_1$ is the bacterial population at time 1 (θ_1) and $\log N_2$ is the bacterial population at time 2 (θ_2) and

$$D = D_0(10)^{(T_0 - T)/z} \qquad (2)$$

where D_0 is the reference decimal reduction time at temperature T_0 and D is the D-value at any temperature T. Regression equations for data from each heat treatment were analyzed separately since processing times for each thermal treatment were different.

RESULTS AND DISCUSSION

The heat resistance of L. monocytogenes in crabmeat heated at 50 to 60°C as expressed as D values ranged from 40.43 to 2.61 min when TSA was used as the plating medium (Table 1). D-values derived from

Table 1. Thermal death times of L. monocytogenes in crabmeat.

Temperature (°C)	D value[a] (min) obtained from	
	Trypticase Soy Agar	Modified Vogel-Johnson Agar
50	40.43	34.48
55	12.00	9.18
60	2.61	1.31

[a] Predicted value based on linear regression.

listerial counts on modified Vogel-Johnson agar were lower and ranged from 34.48 to 1.31 min for the same thermal conditions. The differences in the D-values obtained from the two media at respective times is due to the ability of heat-injured listerial cells to recover on the TSA during the incubation period. Due to the selective ingredients in the modified Vogel-Johnson agar, injured cells did not recover at the same efficiency.

The thermal death time curve for L. monocytogenes as determined using both plating media is shown in Fig. 1. The thermal death time is greater when data from the nonselective rather than the selective growth medium was used (z-value of 8.40 vs. 6.99°C). Again this can be attributed to the difference in the ability of injured Listeria to grow on the different substrates.

The D-values found for thermally-processed crabmeat are similar to values reported by Farber (4) for ground meat but slightly lower. The only description of the meat used in the earlier study was that it was a minced, sausage type meat. Thus speculation concerning the differences in thermal resistance of L. monocytogenes due to the difference in the heating substrate used in the two studies is not possible. Farber also reported D-values in the ground meat product with added curing salts that were 5-8 times greater than that obtained from product free of curing agents.

Concern over possible heat resistance of L. monocytogenes in pasteurized crabmeat is not warranted. The current pasteurization process for crabmeat in steel and aluminum cans is the equivalent of

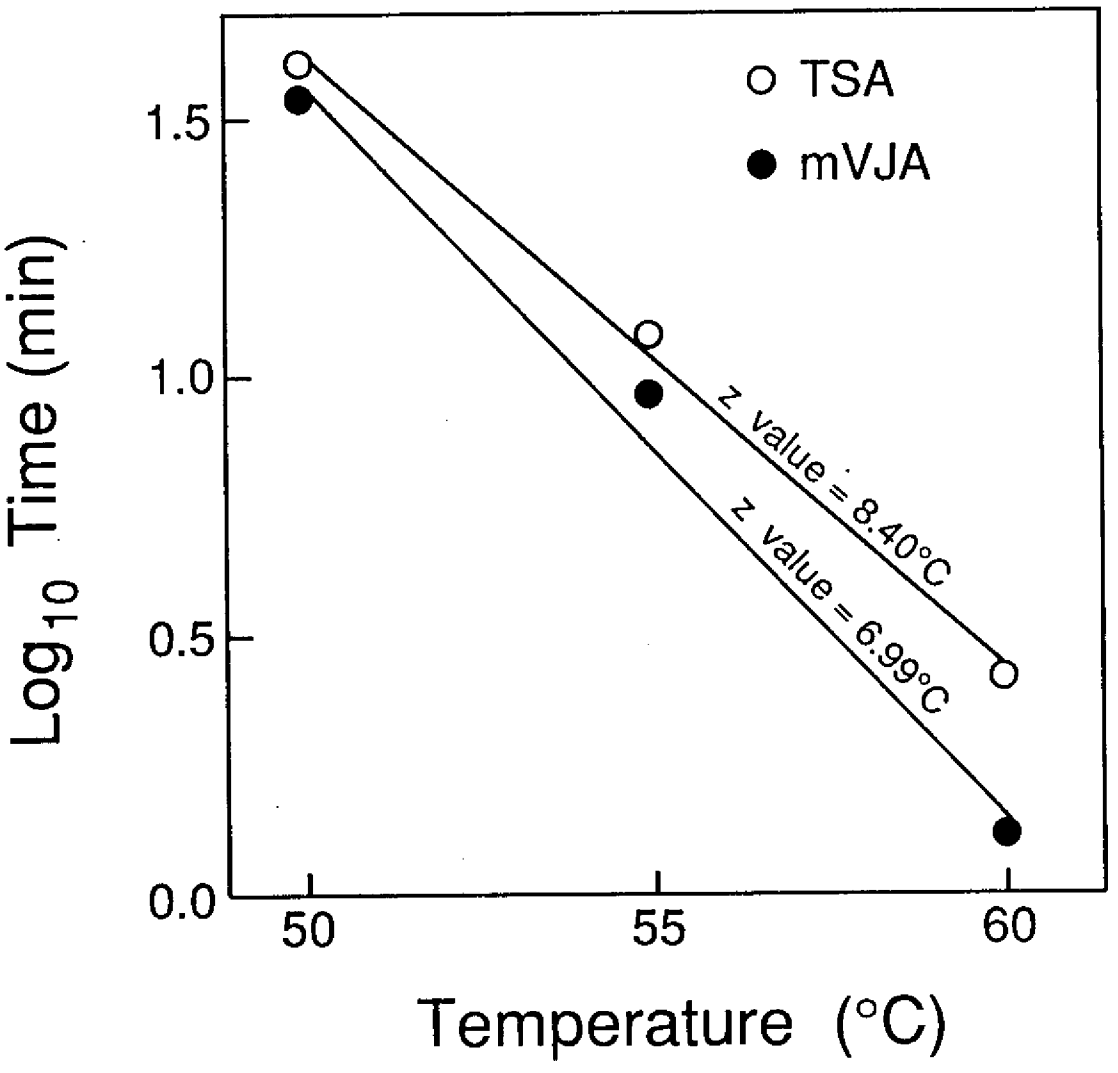

Fig. 1. Thermal destruction curve for L. monocytogenes in crabmeat on trypticase soy agar (TSA) and modified Vogel-Johnson agar (mVJA).

31 min at 185°F (85°C) with a z-value of 16°F (8.89°C) (11). Based on the heat resistance of L. monocytogenes demonstrated in this study, the commercial process would provide a D-process of several hundred fold. This process would seem appropriate even when considering possible differences in crabmeat formulations and considering the problem of heat injured cells.

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EFFECT OF PACKAGING ON THE QUALITY OF ICED FISH

Kouakou Kouadio[1], Yao-wen Huang[1] and Keith Gates[2]

[1]Department of Food Science and Technology, University of Georgia, Athens, GA 30602.
[2]Marine Extension Service, University of Georgia, Brunswick, Georgia 31523.

INTRODUCTION

The U.S. commercial landings (edible and industrial) of fish was worth approximately 2.02 billion dollars in 1988. This was an increase of almost 4 million dollars in value compared with 1987 (USDOC, 1989). Although no one knows the true figures, there may have been at least 30% loss due to improper handling and poor processing (Thoroughgood, 1982). This loss in primarily due to spoilage with some loss due to physical damage. Bacterial induced spoilage and oxidative rancidity are by far the most important causes of fresh fish spoilage. Both mechanisms cause the onset of off-flavors and off-odors, rendering a product of poor quality. Enzymatic activity is also a significant cause of quality deterioration since the activity can proceed at low temperatures even though at slower rate. In addition, fresh fish lacks eye-appeal to the consumers. Many negative attributes such as "slimy" and "smelly" are always associated with fish. Improved packaging of seafoods was listed as a national priority in the Seafood Science and Technology Workshop conducted in May 1982 (Thoroughgood, 1982).

Overwrapping fish in a shallow tray is the oldest method of packaging fish with plastics. This method has been widely investigated by researchers and has always appeared to have little or no effect on shelflife of fish (Davis, 1973; Phillips et al., 1977; Ahvenainen and Malkki, 1985). Vacuum packaging of seafood products, however, proved effective in every single study undertaken. Vacuum packaging in polyethylene bags with low oxygen permeability has been found to substantially reduce oxidative deterioration in fish and fish products (Moyer, 1960; Hardy and Hobbs, 1968; Yu et al., 1973; Lindsay, 1977; Bilinski et al., 1978; Morris and Dowson, 1979) and to slow microbial growth (Daniels et al., 1986). There seems to be no report in the literature about the effectiveness of skin packaging using surlyn films except Lindsay (1977) who reported that high vacuum nozzle evacuated bags suppressed oxidative rancidity better than did film-to-film skin package of surlyn made under low vacuum conditions.

The objective of this study was to investigate the effect of Trigon oxygen permeable Intact Skin Packaging Film (ISPF) in comparison with Cryovac E bag and Saran wrap on the shelflife of weakfish stored in ice for 21 days.

MATERIALS AND METHODS

Collection of fish

One species of fish was used in all sections of this study: weakfish (Cynosion regalis). Fresh fish were harvested by net, off the coast of Brunswick, Geogia, USA. Upon harvest, all fish were deheaded, eviscerated, washed with seawater and placed on ice in ice chests for transportation to the Marine Extension Service warehouse in Bunswick.

Packaging treatment and storage

Handling and preparation of samples were conducted to simulate practices that might be used in commercial fish houses to minimize product damage and microbial contamination. The fish averaged 100 grams in weight. Samples of each treatment were placed on polystyrene trays (2 fish per tray) and were packed by one of the packaging methods described: (1) Overwrapping: samples were overwrapped with Saran wrap (PVDC film); (2) Vacuum packaging: samples were vacuum sealed in Cryovac E oxygen permeable bags (Cryovac Div., Duncan, SC) using a multivac AG 900 vacuum packaging machine (Multivac Sepp Haggenmüller KG, West Germany) followed by a heat shrink process which required the dipping of vacuum packed fish in hot water (190°F) for 2 seconds as specified by Teixeira et al.(1986); (3) Vacuum skin packaging: samples were stretch wrapped sealed film to tray with Trigon oxygen permeable Intact Skin Packaging Film (Trigon Packaging Corporation, Redmond, WA) using a Trigon Intact RM331 Mark III Mini Intact machine.

Samples of each treatment were placed on ice in ice chests immediately after packaging. Specifications of each packaging film are listed in Table 1.

All treatments involved holding fish at about 2:1 ice to fish ratio. Fish were stored in ice chests provided with holes for drainage. Ice chests were transported to the Department of Food Science and Technology, the University of .paGeorgia at Athens, GA and housed in a refrigerated room at 40°F to slow ice melt.

Quality evaluation

Iced samples were removed and evaluated after 3, 7, 10, 14, 17 and 21 days of storage for chemical, microbiological, and sensory attributes. Chemical tests consisted of determining thiobarbituric acid (TBA) number as an index of oxidative rancidity, pH value and ammonia concentration. Microbiological evaluation was determined by total aerobic psychrotrophic count. Sensory quality was assessed by a trained panel to judge sample odor, firmness, and overall acceptance of raw fish. The moisture and free fatty acid contents were monitored as well. Quality evaluation of raw fish was performed on triplicate samples.

Chemical analysis

pH value:

Twenty grams of minced fish flesh was blended using a food processor (Regal La Machine I) with 80 ml of distilled water

Table 1. Physical properties of materials used in packaging treatments.

Packaging materials	Thickness (mil)	Oxygen Transmission Rate cc/100 sq. in/24 hr/atm	Water vapor Transmission Rate gm/100 sq. in/ 24hr/atm
Saran wrap	0.50	0.90 (at 78°F)	0.20 (at 100°F)
Cryovac E bag	2.40	2.58 (at 73°F)	0.65 (at 100°F, 100% RH)
Trigon ISPF	0.20	0.45 (73.4°F, 79% RH)	0.002 (73.4°F, 79% RH)

for 1 minute. The homogenate was filtered over glass wool to collect 50 ml of filtrate. The pH of the filtrate was measured at room temperature with a Fisher Accumet Model 950 pH Meter with a glass electrode.

Ammonia content:

Fifty ml of homogenate were obtained as in pH measurement. Using an Ammonia Ion Selective Electrode coupled into a Fisher Accumet Model 950 pH Meter, millivolt readings were obtained while agitating the solution with a teflon coated magnetic stirring bar and pipetting 1 ml of 10N NaOH into the solution. The NaOH drives ammonia ions (NH_4^+) to ammonia (NH_3), a gas for which the membrane of the electrode is permeable. Ammonia concentrations were determined from a standard curve plotting millivolt readings versus known concentrations of ammonium chloride (Ward et al.,1978). Results were expressed in log millimolar concentration.

TBA number:

To determine 2-thiobarbituric acid (TBA) numbers, the distillation method of Tarladgis et al.(1960) was used with some modifications. Antioxidant solution (200 mg of butylated hydroxy toluene, BHT, in 1.8 g of propylene glycol) was added to the sample before blending to prevent further oxidation as suggested by Yu and Sinnhuber (1967). Instead of 90% glacial acetic acid, distilled water was used to make the 0.02M 2-thiobarbituric acid solution. Absorbances (A) of the TBA-malonaldehyde chromagens against a blank were read at 538 nm on a Bausch and Lomb Spectronic 20 Spectrophotometer. Results were expressed in TBA # = A x 7.8 = mg of malonaldehyde per 100 g of sample.

Moisture content

Approximately 10 g samples were dried to constant weight at 95-100°C under pressure ≤ 100 mm Hg for 5 hours. Loss in weight was reported as percent moisture (AOAC, 1984). .pa

Free fatty acids

Lipids were extracted from fish samples by mixed solvent based on the method of Bligh and Dyer (1959). A separatory funnel was used to separate the filtrate instead of a graduated cylinder. The mixed oil was then titrated as outlined in the AOAC (1984) method 28.032 (a). Amount of 0.25N NaOH used was reported as percent free fatty acids expressed as oleic acid.

Microbiological analysis

Triplicate ten gram samples were obtained aseptically and homogenized for 30 sec with 90 ml sterile Butterfields phosphate buffer in Fisher brand polyethylene sample bags using a Tekmar Stomacher Lab-Blender 400. Serial dilutions and plated onto Standard Methods Agar by the spread plate method. Psychrotrophic counts were determined by counting the colony forming units after the plate were incubated at 7°C for 10 days (Speck, 1984).

Sensory analysis

Six sessions were held on day 3, 7 ,10 ,14 ,17 and 21 of storage. At each sampling raw fish was examined for

odor, firmness and overall acceptability using a 9-point hedonic scale.

Six panelists composed of laboratory technicians and graduate students were selected from the Department of Food Science and Technology of the University of Georgia. Panel members were trained in the basic concepts of affective and descriptive analysis and terminology prior to the study. Fresh fish were purchased from a local retail seafood store and stored in ice for 21 days. During that period, fish were sampled and frozen every 3 days. Panelists were famliarized with the characteristics of fish at every stage of spoilage.

Panelists were seated in individual booths supplied with a reference key (Table 2) and a score sheet (Table 3) and were asked to give a score of 9 (excellent) to 1 (inedible) to rate firmness and odor characteristics of weakfish by marking the number associated with the appropriate descriptor.. Panel members scored the acceptability of samples based on color, odor and firmness attributes on a 9-point hedonic scale. Limit of acceptability was reached when the average sensory rating for either of the descriptors dropped to a value of 5.

Fish were removed from the package, placed in disposable plates, given three digit random numbers and evaluated immediately. Some fish were kept frozen to serve as reference. Sessions were held at 11:00 am in the Food Science taste panel room equipped with separate booths for each panelist. The booths were illuminated with ordinary fluorescent lighting to avoid masking color differences within samples.

Statistical analysis

Statistical analysis (SAS Inst. Inc., 1987) were performed on chemical, sensory, microbiological, and moisture and FFA contents data by means of PC SAS. The General Linear Regression procedures and Duncan's multiple range test were used to determine any significant differences among days of storage and package materials stored in ice. Linear regression was performed on data to investigate significant relationships among measured sensory, chemical, microbiological, and moisture and FFA contents parameters.

RESULTS AND DISCUSSIONS

A Chemical Analysis

The results of chemical analyses of the pH and ammonia content for weakfish packaged in ISPF, E bag and Saran wrap are shown in Tables 4 and 5 respectively. The Trends of ammonia resemble that of pH and the values of both parameters increased little during the first 17 days, but increased markedly thereafter. Thus, the increase in pH and ammonia content was delayed by packaging up to 17 days. In fact, some investigators observed an earlier increase of these parameters with unpackaged iced dogfish (Bilinski et al., 1983; Rasevi et al., 1985). Packaging treatments were not significantly different ($p>0.05$) for changes in pH and

Table 2. Reference key for raw fish evaluation

Instructions

- Avoid exposure to foods and fragrance for 30 minutes before panels.
- Leave 20 to 30 seconds between sample evaluations to reduce sensory adaptation.
- Do not smoke at least 30 minutes before session starts.

Measuring scales

Firmness: 1 - soft to 9 - extremely firm

Odor : 1 - extremely off odor to 9 - extremely fresh odor

Overall acceptability: 1 - unacceptable to 9 - excellent

Definition of terms

Firmness: force required to press the skin of fish with the middle finger.

Odor: the characteristic smell of the product.

Overall acceptability: degree of acceptance of the product.

Table 3. Score sheet for raw fish rating

Name:_____ Date:_____

Please evaluate each sample for each descriptor listed below.

	Reference	S a m p l e s		
		_____	_____	_____
Firmness	_____	_____	_____	_____
Odor	_____	_____	_____	_____
Overall acceptability	_____	_____	_____	_____

Table 4. Analysis of variance and mean values for pH of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	6.92^{b}_{a}	6.88^{d}_{b}	6.93^{b}_{a}	16.30	0.0038
7	7.00^{b}_{b}	7.04^{bc}_{a}	6.99^{b}_{b}	29.40	0.0008
10	7.01^{b}_{a}	6.97^{cd}_{a}	7.00^{b}_{a}	0.12	NS[2]
14	7.09^{b}_{a}	7.02^{bc}_{b}	6.98^{b}_{c}	20.73	0.0020
17	7.03^{b}_{a}	7.10^{b}_{a}	7.05^{b}_{a}	1.10	NS
21	7.31^{a}_{a}	7.56^{a}_{a}	7.62^{a}_{a}	2.97	NS
Std Dev.	0.15	0.23	0.25		

[1]Results are the means of three pH values for each sample. Means with the same superscript in the same column are not significantly different at $\alpha = 0.05$ level.
For each row, means with the same subscript are not significantly different at $\alpha = 0.05$ level.

[2]NS = not significant

Table 5. Analysis of variance and mean values for ammonia concentration of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	0.99^{d}_{a}	0.92^{d}_{c}	0.95^{d}_{b}	13.79	0.0057
7	1.95^{cd}_{a}	1.03^{d}_{b}	0.99^{d}_{b}	25.21	0.0012
10	3.02^{c}_{a}	2.32^{c}_{a}	2.43^{c}_{a}	3.70	NS[2]
14	2.42^{cd}_{a}	1.90^{c}_{b}	2.54^{c}_{a}	5.83	0.0392
17	4.57^{b}_{a}	3.59^{b}_{a}	4.26^{b}_{a}	1.45	NS
21	9.64^{a}_{a}	7.12^{a}_{a}	7.90^{a}_{a}	2.92	NS
Std Dev.	3.00	2.21	2.51		

[1]Results are the means of three ammonia concentrations for each sample. Means with the same superscript in the same column are not significantly different at $\alpha = 0.05$ level.
For each row, means with the same subscript are not significantly different at $\alpha = 0.05$ level.
Values are expressed in log millimolar concentration.

[2]NS = not significant

Table 6. Analysis of variance and mean values for TBA value of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	0.17^{C}_{a}	0.18^{C}_{a}	0.14^{C}_{a}	0.21	NS[2]
7	0.36^{C}_{a}	0.33^{C}_{a}	0.22^{bc}_{a}	2.99	NS
10	0.47^{C}_{a}	0.94^{bc}_{a}	0.53^{ab}_{a}	0.88	NS
14	1.05^{b}_{a}	1.18^{ab}_{a}	0.39^{abc}_{b}	7.57	0.0229
17	1.79^{a}_{a}	1.71^{a}_{a}	0.39^{abc}_{b}	23.16	0.0015
21	1.73^{a}_{a}	1.75^{a}_{a}	0.64^{a}_{b}	7.83	0.0213
Std Dev.	0.71	0.71	0.21		

[1]Results are the means of three TBA values for each sample. Means with the same superscript in the same column are not significantly different at $\alpha = 0.05$ level.
Means with the same subscript for each row are not significantly different at $\alpha = 0.05$ level.
Values are expressed in mg of malonaldehyde/1000 g of sample.

[2]NS = not significant

Table 7. Analysis of variance and mean values for psychrotrophic bacteria counts on weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	1.96^{f}_{b}	2.36^{f}_{a}	2.18^{f}_{ab}	3.53	NS[2]
7	3.33^{e}_{a}	3.37^{e}_{a}	2.95^{e}_{b}	6.78	0.0289
10	4.02^{d}_{a}	3.97^{d}_{a}	3.84^{d}_{a}	1.09	NS
14	5.52^{c}_{b}	6.46^{c}_{a}	5.40^{c}_{b}	8.63	0.0172
17	6.65^{b}_{b}	7.43^{b}_{a}	6.63^{b}_{b}	28.22	0.0009
21	7.65^{a}_{a}	8.01^{a}_{a}	7.72^{a}_{a}	1.89	NS
Std Dev.	2.01	2.20	2.05		

[1]For each column, means with the same superscript are not significantly different at $\alpha = 0.05$ level. Likewise, means with the same subscript for each row are not significant at $\alpha = 0.05$ level.
Values are expressed in logarithmic unit per 10 g sample.

[2]NS = not significant

Table 8. Analysis of variance and mean values for free fatty acids content of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	0.83^{c}_{b}	0.95^{c}_{a}	0.88^{b}_{ab}	4.63	NS[2]
7	0.90^{c}_{b}	0.95^{c}_{b}	1.03^{ab}_{a}	11.69	0.0085
10	1.07^{b}_{a}	0.98^{c}_{ab}	0.92^{b}_{b}	6.78	0.0289
14	1.13^{b}_{a}	1.15^{b}_{a}	1.05^{ab}_{a}	0.85	NS
17	1.55^{a}_{a}	1.35^{a}_{ab}	1.20^{a}_{b}	6.17	0.0351
21	1.48^{a}_{a}	1.32^{a}_{b}	1.20^{a}_{b}	12.88	0.0067
Std Dev.	0.28	0.18	0.16		

[2]Means with the same superscript for each column are not significantly different at $\alpha = 0.05$ level.
Means with the same subscript for each row are not significantly different at $\alpha = 0.05$ level.
Values are measured in percent free fatty acids expressed as oleic acid.

[2]NS = not significant

Table 9. Analysis of variance and mean values for percent moisture content of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	76.92^{a}_{a}	77.85^{abc}_{a}	77.33^{a}_{a}	2.19	NS[2]
7	77.59^{a}_{a}	76.05^{c}_{a}	76.03^{a}_{a}	1.86	NS
10	76.98^{a}_{a}	77.03^{bc}_{a}	76.97^{a}_{a}	0.01	NS
14	76.97^{a}_{a}	78.18^{ab}_{a}	77.02^{a}_{a}	3.64	NS
17	77.70^{a}_{a}	79.37^{a}_{a}	77.29^{a}_{a}	1.38	NS
21	76.82^{a}_{b}	78.71^{ab}_{a}	77.48^{a}_{ab}	3.32	NS
Std Dev.	1.02	1.39	0.86		

[1]Means with the same superscript for each column are not significantly different at $\alpha = 0.05$ level.
Means with the same subscript for each row are not significantly different at $\alpha = 0.05$ level.
Values are expressed as percent moisture.

[2]NS = not significant

(1986) also observed no change in moisture content of samples during 1 to 4 weeks trial at 2°C.

E Sensory Analysis

Tables 10, 11 and 12 summarize the sensory scores as a function of iced storage time of weakfish. There was no change in firmness scores, which indicated that weakfish texture remained unchanged with storage time. However, a progressive deterioration in odor, as indicated by a decrease in odor scores, was observed. The overall desirability scores decreased with storage time as well, and all samples were unacceptable on day 17. In addition, panelists were unable to differentiate among samples from all packaging treatments for odor, texture (firmness) and overall desirability during the 21 days of iced storage. Nevertheless, packaging extended the shelf life of fish. Under similar conditions, unprotected fish lost their quality on day 12 (Bilinski et al., 1983). Stroud et al. (1982) observed a sensory shelflife of 8 to 11 days in unpackaged fish.

F Correlation of Spoilage Indicators

Tables 13, 14 and 15 present the correlations between chemical, microbiological, sensory, moisture and FFA content parameters for three different packaged samples. Results showed high correlations between ammonia content and pH for samples packed in E bag ($r = 0.86$), in Saran wrap ($r = 0.90$) and in ISPF ($r = 0.87$). Rasevi et al. (1985) found a correlation of 0.84 between these two parameters. This high correlation confirms the statement that a high marine fish pH results from basic compounds such as ammonia and trimethylamine (Stroud et al., 1982; Bilinski et al., 1983). Thus, pH measurement showed potential as a simple, rapid method for monitoring ammonia content. There was also high correlations between ammonia content and sensory scores for odor ($r = 0.92$ for ISPF) and overall acceptability ($r = 0.88$ for ISPF). Therefore, ammonia could be a useful index for the objective assessment of fish quality (Vyncke, 1968). The high correlations observed between ammonia and bacteria counts ($r=0.84$ for ISPF) indicated that ammonia production may be related to bacterial enzyme activity. TBA value which is a measure of oxidative rancidity was in good correlation with FFA and APC for samples packaged in Saran wrap and E bag. However, these parameters correlated little when fish were vacuum-skin packed in ISPF. In E bag and saran wrap lipid hydrolysis may be readily affected by microbial enzyme and results in FFA which are prone to oxidative rancidity. FFA and pH did not correlate well ($r=0.42$ for ISPF; $r=0.49$ for Saran wrap; $r=0.45$ for E bag). Therefore, the increase in FFA during short-term storage could not affect the pH as observed by Bilinski et al.(1983). Sensory odor is an important parameter in judging fish quality. More often, panelists in rating overall acceptability rely heavily on odor characteristics. In this study, extremely high correlations were found between odor and overall desirability ($r=0.95$ for ISPF;

Table 10. Analysis of variance and mean values for firmness of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	7.55^{a}_{a}	7.33^{a}_{a}	7.67^{a}_{a}	0.29	NS[2]
7	7.83^{a}_{a}	7.50^{a}_{a}	7.33^{a}_{a}	0.39	NS
10	7.33^{a}_{a}	7.67^{a}_{a}	8.00^{a}_{a}	2.00	NS
14	7.18^{a}_{a}	6.67^{a}_{a}	7.00^{a}_{a}	1.21	NS
17	6.33^{a}_{a}	6.50^{a}_{a}	6.33^{a}_{a}	0.03	NS
21	7.00^{a}_{a}	6.17^{a}_{b}	6.83^{a}_{a}	5.00	0.0312
Std Dev.	1.24	1.28	1.39		

[1]For each column, means with the same superscript are not significantly different at $\alpha = 0.05$ level.
For each row, means with the same subscript are not significantly different at $\alpha = 0.05$ level.

[1]NS = not significant

Table 11. Analysis of variance and mean values for odor of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	7.17^{a}_{a}	7.33^{a}_{a}	7.17^{a}_{a}	0.45	NS[2]
7	6.67^{a}_{ab}	6.00^{ab}_{b}	6.83^{a}_{a}	3.89	NS
10	6.17^{a}_{a}	5.83^{ab}_{a}	6.17^{a}_{a}	0.19	NS
14	5.59^{a}_{a}	5.00^{bc}_{a}	5.50^{a}_{a}	1.15	NS
17	3.33^{b}_{a}	4.00^{cd}_{a}	3.67^{b}_{a}	0.24	NS
21	2.83^{b}_{a}	2.50^{d}_{a}	2.50^{b}_{a}	0.09	NS
Std Dev.	2.34	2.16	2.18		

[1]For each column, means with the same superscript are not significantly different at $\alpha = 0.05$ level.
For each row, means with the same subscript are not significantly different at $\alpha = 0.05$ level.

[2]NS = not significant

Table 12. Analysis of variance and mean values for overall acceptability of weakfish stored 3 weeks in 3 different packaging materials.[1]

Days of storage	Treatments			F-value	Prob>F
	Cryovac E bag	PVDC Saran	Trigon ISPF		
3	7.83^{a}_{a}	7.83^{a}_{a}	7.67^{a}_{a}	0.12	NS[2]
7	7.00^{a}_{a}	6.50^{ab}_{a}	7.17^{a}_{a}	1.10	NS
10	6.17^{a}_{a}	6.50^{ab}_{a}	7.17^{a}_{a}	1.04	NS
14	5.83^{a}_{a}	4.83^{bc}_{a}	5.33^{b}_{a}	1.67	NS
17	3.17^{b}_{a}	3.83^{cd}_{a}	4.50^{b}_{a}	1.08	NS
21	3.00^{b}_{a}	2.33^{d}_{a}	2.83^{c}_{a}	1.00	NS
Std Dev.	2.55	2.44	2.07		

[1]For each column, means with the same superscript are not significantly different at $\alpha = 0.05$ level.
For each row, means with the same subscript are not significantly different at $\alpha = 0.05$ level.

[2]NS = not significant

Table 13. Linear regression for data obtained with Cryoac E bag.

Regression	correlation coefficient	Slope	Intercept
pH vs. ammonia	0.86	21.54	-148.33
pH vs. bacteria count	0.70	13.37	-88.54
Ammonia vs. odor	0.78	-0.51	7.22
Ammonia vs. accepta.	0.74	-0.55	7.57
Ammonia vs. count	0.73	0.58	3.65
TBA vs. odor	0.96	-2.48	7.60
TBA vs. accepta.	0.95	-2.75	8.05
TBA vs. count	0.93	2.92	3.15
Odor vs. accepta.	0.99	1.10	-0.34
Odor vs. count	0.92	-1.14	11.89
Firmness vs. accepta.	0.72	3.28	-18.09
Accepta. vs. count	0.93	-1.03	11.52
pH vs. FFA	0.45	1.49	-9.33
TBA vs. FFA	0.96	0.41	0.78
Odor vs. FFA	0.96	-5.95	12.20
Accepta.vs. FFA	0.98	-6.67	13.25
Count vs. FFA	0.89	6.82	-2.06

Table 14. Linear regression for data obtained with PVCD (Saran wrap)

Regression	correlation coefficient	Slope	Intercept
pH vs. ammonia	0.90	9.21	-62.55
pH vs. bacteria count	0.56	7.28	-45.40
Ammonia vs. odor	0.85	-0.67	7.00
Ammonia vs. accepta.	0.80	-0.78	7.50
Ammonia vs. count	0.65	0.81	3.98
TBA vs. odor	0.86	-2.35	7.50
TBA vs. accepta.	0.87	-2.84	8.18
TBA vs. count	0.94	3.39	2.82
Odor vs. accepta.	0.98	1.19	-0.76
Odor vs. count	0.90	-1.31	12.97
Firmness vs. accepta.	0.83	3.02	-15.77
Firmness vs. count	0.84	-3.50	30.85
Accepta. vs. count	0.95	-1.13	12.24
pH vs. FFA	0.49	0.54	-2.72
Ammonia vs. FFA	0.63	0.06	0.94
TBA vs. FFA	0.89	0.26	0.85
Odor vs. FFA	0.81	-8.24	14.31
Accepta.vs. FFA	0.87	-10.22	16.71
Count vs. FFA	0.92	12.16	-7.31

Table 15. Linear regression for data obtained with Trigon oxygen permeable ISPF.

Regression	correlation coefficient	Slope	Intercept
pH vs. ammonia	0.87	9.38	-63.38
pH vs. bacteria count	0.56	6.21	-38.26
Ammonia vs. odor	0.92	-0.68	7.47
Ammonia vs. accepta.	0.88	-0.68	7.95
Ammonia vs. count	0.84	0.76	3.37
TBA vs. odor	0.57	-7.53	8.20
TBA vs. accepta.	0.50	-7.22	8.56
TBA vs. count	0.60	9.02	2.31
Odor vs. accepta.	0.95	0.99	0.47
Odor vs. count	0.96	-1.14	11.86
Firmness vs. accepta.	0.62	2.47	-11.99
Firmness vs. count	0.60	-2.77	25.77
Acceptab.vs. count	0.96	-1.12	12.24
pH vs. FFA	0.42	0.34	-1.36
TBA vs. FFA	0.27	0.38	0.90
Odor vs. FFA	0.81	-12.36	18.23
Accepta.vs. FFA	0.83	-12.77	19.15
Count vs. FFA	0.81	14.48	-9.37

r=0.99 for E bag; r=0.98 for Saran wrap). Bremner et al. (1988) found a correlation coefficient of 0.82 between the two parameters. The deterioration of odors in fish is very often accompanied by an increase in TBA number. Several researchers have observed significant correlation between sensory scores and TBA values (Angel et al., 1981; Ahvenainen et al.,1985; Josephson et al., 1985; Kurade et al., 1987; Jahncke, 1989). For this investigation, the correlations were 0.96 between TBA and odor, and 0.95 between TBA and overall acceptability in E bag packed samples.

CONCLUSION

Under iced storage, packaging treatments were not significantly different for pH and ammonia changes in weakfish up to 17 days. Therefore, the type of film and method of packaging did not have a significant influence on the change in ammonia content and pH of weakfish flesh. Compared to the E bag and overwrap, ISPF significantly suppressed the development of rancidity in weakfish over the 21 days of storage. ISPF significantly ($p<0.05$) slowed lipid hydrolysis compared to E bag, but was not statistically different from Saran overwrap. Moisture migration through the films was found to be neglibible for all packages tested in this study. ISPF and E bag were more effective than Saran overwrap in delaying bacterial growth. Sensory analysis revealed that packaging treatments were not significantly different for firmness, odor, and overall acceptability of weakfish. Sensory analysis, along with microbiological assessment of raw samples, limited the shelflife to 14-17 days for weakfish packaged in E bag and ISPF, and not more than 14 days for Saran overwrapped fish.

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IDENTIFICATION AND COMPARISON OF VOLATILE ODOROUS COMPOUNDS IN POLLACK AND MENHADEN SURIMIS

Charles F. Lin* and Thomas C.-Y. Hsieh

Department of Food Science, Louisiana Agric. Expt. Station, LSU Agricultural Center and Louisiana Sea Grant College Program, Louisiana State University and A&M College, Baton Rouge, LA 70803-4200. *Current address: Calreco, Inc., NESTEC Ltd., Van Nuys, CA 91412.

INTRODUCTION

Surimi is a processed form of minced, washed and strained fish flesh. In recent years, seafood analogs based on surimi have gained wide acceptance from U.S. consumers. Alaska pollack (Theragra chalcogramma) has been the major species for manufacturing surimi products in the U.S. (Babbitt, 1986). However, menhaden represented 91% of U.S. commercial fishery landings in 1986 and 1987 and has been used primarily in low-valued fish oil and fish meal production (Bimbo, 1988). Therefore, the menhaden fishery industry has been interested in evaluating the suitability of this underutilized marine species for surimi production. Zapata Haynie Co. has produced menhaden surimi on an experimental basis to make available for evaluation by researchers (Bimbo, 1987). A desirable surimi should be bland without introducing any appreciable odors to the final products. Unfortunately, menhaden surimi has been noted to contain undesirable odors (Hsieh, 1989). Machii et al. (1987) indicated that undesirable odors in surimi should be removed or masked.

The objectives of this study were to identify and to compare the volatile components in Alaska pollack and Atlantic menhaden surimis.

MATERIALS AND METHODS

Menhaden fish and surimi: Menhaden fish (Brevoortia tyrannus) were harvested in 1988 from the Atlantic Ocean by Zapata Haynie Corp. (Reedville, VA). Menhaden surimi samples were prepared by Zapata Haynie Corp.

Pollack surimi: Pollack (Theragra chalcogramma) surimi samples were obtained from the Northwest and Alaska Fisheries Center, National Marine Fisheries Service, Kodiak, AK.

Shipment of surimi: Frozen menhaden and pollack surimi samples in plastic bags were packed in a box filled with dry ice and shipped by next-day air-cargo.

Sample preparation: Frozen surimi samples were cut into slices (6 cm x 6 cm x 0.8 cm) and cooked in a microwave oven (Thermador CP55, Los Angeles, CA) at 70% of full power (650 x 70% = 455 watts) to an internal temperature of 150°F and held at 150°F for 4 min.

Flavor standards: Flavor standards were purchased from Aldrich Chemical Co. (Milwaukee, WI), Pfaltz & Bauer, Inc. (Waterbury, CT), Fluka Chemical Corp. (Ronkonkoma, NY), and Tokyo Kasei (Portland, OR). Some flavor standards were gifts from the Flavors and Fragrances Division of Aldrich Chemical Co.

Dynamic headspace sampling (DHS): System blanks were analyzed prior to sample analysis under comparable conditions to ensure that DHS/GC/MS was free from contamination or procedural artifacts. Sixteen grams of cooked surimi were diced into 0.25-cm cubes and transferred into 4 purge tubes. The tubes were kept on ice before purging. Volatile components in the surimi were collected on a dynamic headspace sampler consisting of a Tekmar 4200/4000 (Cincinnati, OH) system. Each tube was pre-purged for 0.5 min at ambient temperature with helium gas (99.999%) at a flow rate of 40 mL/min to remove any oxygen inside the sample tube. The sample was then heated and maintained at 65°C for 30 min to allow the volatile components to be purged and absorbed onto a trap cartridge containing Tenax TA (2,6-diphenyl-p-phenylene oxide polymer, 0.3g, 60/80 mesh, Chrompack Inc., Raritan, NJ). The Tenax TA trap was initially conditioned at 340°C for 2 hr with a helium flow of 30 mL/min and baked at 225°C for 10 min immediately before sample purging to ensure no artifacts from the trap and no carry-over of volatiles from previous samples. An off-line dry purge was carried out for 3 hr at a flow rate of 40 mL/min of helium gas to remove moisture absorbed inside the trap (Williams et al., 1988). Volatiles trapped in the Tenax TA were then flash-desorbed at 185°C for 15 min with a helium flow of 40 mL/min, and cryogenically (liquid nitrogen) focused in a fused silica capillary column prior to chromatography.

Gas chromatography/mass spectrometry (GC/MS): Separation of volatile components was performed on a fused silica capillary column (Supelcowax 10, 60 m length x 0.25 mm i.d. x 0.25 μm film thickness) installed in a Hewlett-Packard (HP) 5792A GC (Palo Alto, CA). Helium gas at a linear velocity of 25 cm/sec was used as the carrier gas. The column temperature was programmed from 27°C, held for 5 min, then increased to 175°C at 1°C/min, further increased to 195°C at 5°C/min, and finally maintained at 195°C for 30 min. The total run time for each sample was 160 min.

Electron ionization mass spectral data acquisition was carried out as described elsewhere (Hsieh et al., 1989). Retention indices were determined according to Van den Dool and Kratz (1963) by using a series of standard n-alkanes (PolyScience Corp., Niles, IL) in separate GC runs. Mass spectral patterns, chromatographic retention indices, and characteristic flavor notes from sniffing of the sample were utilized to match that of samples for identification of volatile components.

GC/photo-ionization detection and odor perception: A Supelcowax 10 column similar to the one used in the GC/MSD was used in a HP 5793 GC equipped with a photo-ionization detector. Other details of this procedure have been described elsewhere (Tanchotikul and Hsieh, 1989).

RESULTS AND DISCUSSION

Seventy-six and 47 compounds were identified by DHS/GC/MS in cooked menhaden and pollack surimis, respectively (Table 1). These compounds included alkanes, alkenes, aldehydes, alcohols, ketones, alkyl-benzenes, heterocyclic and sulfur-containing components. Chromatograms of volatile compounds in microwave-cooked pollack and menhaden surimis are shown in Fig. 1A and 1B, respectively. Menhaden surimi has been observed to have stronger and more undesirable odors than that of pollack surimi (Hsieh, 1989). The majority of the volatile components in menhaden surimi had a larger peak area than that of the same compound in pollack surimi (Fig. 1).

Saturated aldehydes (C4-C9) in the menhaden surimi were observed to have two to eight times larger areas than the corresponding compounds in the pollack sample. Similarly, unsaturated aldehydes, such as (E)-2-pentenal, (E)-2-hexenal and (Z)-4-heptenal, also were found to be two to 11 times higher in the menhaden surimi than in the pollack sample. These compounds were associated with green grassy, oxidized lipid, cucumber-like and a rotten sweet potato note in the samples. In contrast to the above, two branched aldehydes, 2-methylbutanal and 3-methylbutanal were found to be equal or higher in pollack surimi than the counterparts in the menhaden surimi. These two aldehydes imparted mild green with sweet-woody note to pollack surimi. Benzaldehyde contributed an undesirable cherry/fruity flavor to the surimi samples. Desirable surimi, often used as ingredients in seafood analogs, should be a bland ingredient without any appreciable odor. Alkadienals, (Z,Z)-2,4-heptadienal and (E,E)-2,4-heptadienal, were detected and found to be associated with oxidized and oily green odors in menhaden samples but were absent in pollack samples. Certain alkatrienals have been reported to possess fish odors in oxidized oils (Badings, 1970, 1973; Meijboom and

Table 1: Volatile compounds in pollack and menhaden surimis

Peak No	Compound Names	Area % men.	Area % pol.	Area ratio men./pol.
	Aldehydes	**15.5**	**30.0**	
6	2-methylpropanal	0.2	0.8	1
9	butanal	0.9	1.4	2
11	2-methylbutanal	1.2	3.8	1
12	3-methylbutanal	2.4	16.9	<1
17	pentanal	2.1	1.5	3
24	hexanal	4.1	1.9	5
28	(E)-2-pentenal	0.5	0.3	4
31	heptanal	0.6	0.5	1
33	(E)-2-hexenal	0.7	0.2	5
36	(Z)-4-heptenal *	0.6	1.1	1
42	octanal	0.5	0.4	2
54	nonanal	0.4	0.1	<1
65	2,4-heptadienal	0.1	-	+
66	benzaldehyde	1.1	1.1	1
67	2,4-heptadienal	0.1	-	+
	Alcohols	**23.0**	**11.8**	
32	1-penten-3-ol	11.9	8.9	49
34	3-methylbutanol	0.3	0.4	<1
39	1-pentanol	0.3	0.2	9
45	(Z)-2-penten-1-ol *	1.5	-	+
47	(E)-2-penten-1-ol	1.5	1.1	<1
51	1-hexanol	0.1	-	+
54	(E)-3-hexen-1-ol	0.1	-	+
59	1-octen-3-ol	1.4	t	15
60	1-heptanol	0.3	-	+
62	1,5-octadien-3-ol *	3.8	0.9	18
63	2-ethyl-1-hexanol	0.9	0.3	3
68	2,3-butanediol	0.1	-	+
69	1-octanol	0.5	-	+
74	1-nonanol	t	-	+
74	2-undecanol	0.3	-	+
	Ketones	8.9	3.3	
10	2-butanone	0.7	1.4	2
15	3-buten-2-one	0.2	0.1	3
18	2,3-butanedione	3.2	0.8	5
21	3-hexanone	0.1	0.2	<1
22	2,3-pentanedione	3.5	0.8	4
40	cyclohexanone *	0.1	t	+

41	5-methylfuranone	0.1	-	+
52	5-methyl-2-hexanone *	0.2	-	+
61	2,3-nonanedione *	0.4	-	+
70	4-octanone	0.1	-	+
71	3,5-octadien-2-one	0.3	-	+
	Alkanes	**29.5**	**2.4**	
2	hexane	2.3	1.5	10
3	heptane	1.1	0.7	6
5	octane	0.3	0.2	5
19	decane	0.2	t	+
26	undecane	0.1	t	+
43	tridecane	0.2	t	+
54	tetradecane	0.3	t	+
63	pentadecane	14.3	t	+
72	hexadecane	0.9	t	+
73	heptadecane	9.8	t	+
	Alkenes	**3.0**	**1.1**	
4	1-pentene	0.1	-	+
7	4-octene	0.1	-	1
8	2-octene	0.1	-	+
13	3-methyl-1,4-heptadiene *	2.1	1.1	4
25	1,3,6-heptatriene *	0.2	t	+
35	1,3,7-octatriene *	0.2	t	+
76	1-hexadecene *	0.9	t	+
	Benzene derivatives	**3.3**	**2.1**	
14	benzene	0.3	0.6	1
20	toluene	0.4	0.3	3
27	ethylbenzene	0.1	-	+
29	p-xylene	t	-	+
30	m-xylene	0.1	-	+
37	2-ethyltoluene	0.1	0.3	4
38	4-isopropyltoluene	0.6	0.4	3
44	1,3-diethylbenzene	0.1	-	+
46	C3-benzene	0.1	-	+
48	1,2,3-trimethylbenzene	0.3	-	+
56	C4-benzene	0.1	-	+
57	C4-benzene	0.2	-	+
58	1,4-dichlorobenzene	0.7	0.5	4
77	naphthalene	0.2	-	+
	Other compounds	**8.2**	**10.2**	
16	2-ethylfuran	0.6	t	1
23	dimethyl disulfide	7.6	10.2	35

t = percentage below 0.05%.
* = tentative identification based on MS data only.
- = not detected.
+ = Compounds present only in the menhaden surimi.

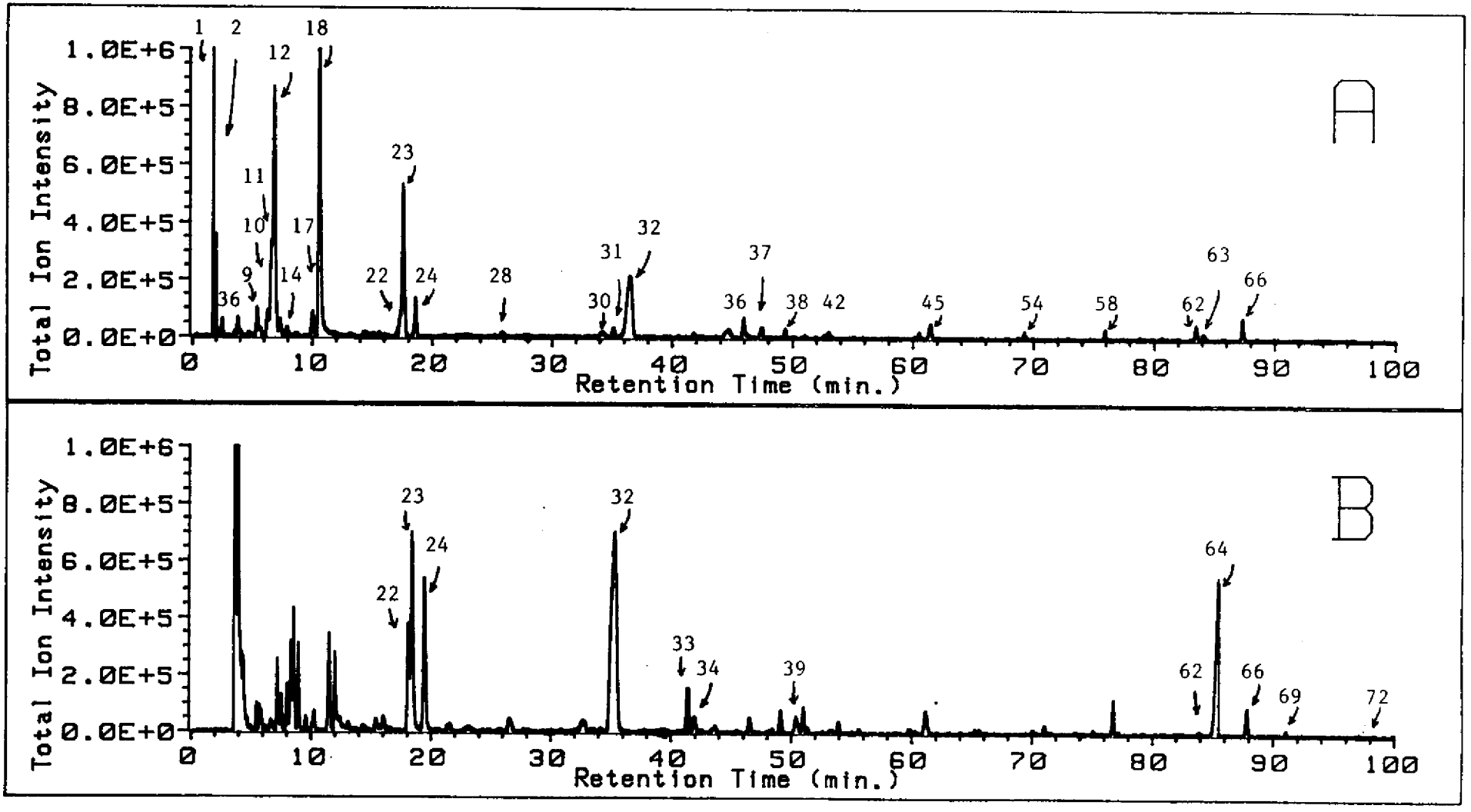

Fig. 1 (A & B) - Total ion chromatograms of volatile compounds analyzed by DHS/GC/MS in cooked (A) pollack and (B) menhaden surimis.

Stroink, 1972; Karahadian and Lindsay, 1988; Hsieh et al., 1989); however, they were not detected in either of the samples studied.

Six alcohols were identified in the pollack surimi with 15 detected in the menhaden surimi sample. Higher amounts of 1-penten-3-ol (49-fold), 1,5-octadien-3-ol (18-fold) and 1-octen-3-ol (15-fold) were found in the menhaden surimi than in the pollack surimi (Table 1). The saturated alcohols (C5-C9) were found in menhaden surimi while only 1-pentanol was detected in the pollack sample. 1-Pentanol had a green woody odor and 2-penten-1-ol contributed a rotten grass odor to the menhaden surimi.

Eleven and six ketones were detected in the menhaden and the pollack samples, respectively. In the cooked surimi, 2,3-butanedione and 2,3-pentanedione gave an undesirable dairy flavor to both menhaden and pollack samples.

While five benzene-containing compounds were found in the pollack surimi sample, 14 compounds were detected in the menhaden surimi. This series of compounds included alkylbenzenes, naphthalene and 1,4-dichlorobenzene, which gave, in general, medicinal, solvent, and pesticide odors to the samples. Contribution of undesirable odors by these compounds in the menhaden surimi was considered much more pronounced than in pollack surimi because of higher amounts of these compounds.

The 2-ethylfuran was found in the dynamic headspace of both the menhaden and pollack surimi samples and contributed a solvent-like odor to samples. Menhaden surimi contained 35 times more 2-ethylfuran in the dynamic headspace than did the pollack surimi. Dimethyl disulfide was the only sulfur-containing compound found in surimi volatiles. This compound gave a cooked/fermented cabbage note and was considered an undesirable odor in both menhaden and pollack surimi samples.

DHS/GC/MS and odor perception with photo-ionization detection at the GC exit port were found to be effective in identification and comparison of volatile flavor components in the surimi samples. This combined analytical approach can be used to monitor the flavor quality of surimi and other seafood products.

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Screening Moisture Binding Agents Which Influence Shrimp Texture and Yields

L.E. Barton, L.A. Applewhite and W.S. Otwell
Food Science Department
University of Florida
Gainesville, Florida

INTRODUCTION

Use of chemical additives is increasing in the seafood industry. These additives are used to serve some definite purpose and perform a wide variety of functions. Chemical additives (i.e. phosphates, isolated soy proteins, various salt blends, etc.) are being used in a wide variety of applications in the processing of seafood for moisture and texture control.

Chemical additives, if used properly, can greatly increase the quality of our seafood products. However, moisture binding agents have been used in some instances without a full appreciation of the influence or consequence of the additives or method of application. Knowledge about processing aids and their methods of application to seafood has been transferred mainly from the beef and poultry industry with limited regard for consequence for seafood quality.

This project was initiated to investigate the effects of chemicals additives and their physical applications on seafood. The ultimate benefits of this project are the efficient utilization and added value of the nation's marine resources for the economic welfare of the seafood industry and satisfaction of consumers. This project is one part of a research project funded by an S-K grant.

METHOD

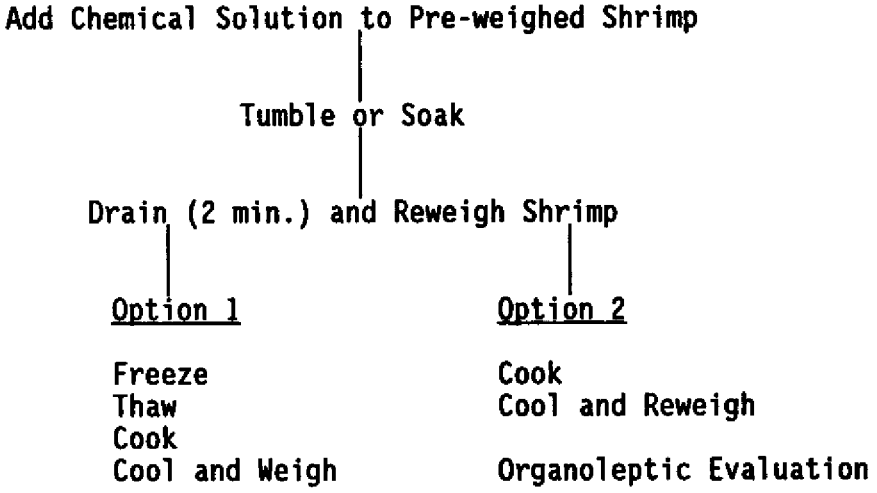
The primary objective for this part of the project was to screen a variety of processing aids (chemicals) for their influence on moisture control, yield and sensory attributes. A standard screening procedure was developed to better understand the proper use of and the influence and consequence of those chemicals used as moisture binding agents and their methods of application (Table 1). Over 60 chemicals have been screened, as well as a variety of shrimp species and sizes.

Table 1. Standard Screening Procedure

1. Vacuum + -
2. Type of Treatment: Soak versus Tumble
3. Treatment Time
4. Chemical Concentration
5. Ratio of Shrimp to Treatment Solution

In testing each chemical and combination of chemicals, a standard testing procedure was followed (Figure 1). Each shrimp sample was weighed and combined with a specific chemical solution and tumbled or soaked for a specified amount of time. Following the application, the treated sample was drained for 2 minutes and weighed. At this point there are two options. Option 1, freeze the treated sample, then thaw and cook at a later date. Option 2, cook the treated sample immediately after treatment. After following option 1 or 2, the sample was cooled, weighed and evaluated organoleptically.

Figure 1. Standard Testing Procedure



This testing procedure provided data at several stages concerning moisture control, drip loss and yield. Product weights were collected after the initial chemical treatment, after the freeze/thaw cycle, and after cooking. Data at each of these stages were extremely important in evaluating the consequence of a particular chemical through all processing stages.

A unique lab scale tumbler (300g capacity) was devised by the authors to monitor the physical actions of tumbling on the shrimp. With the help of a glass blower, a flat bottom, smooth surface flask was modified with indentation to mimic commercial tumblers used in the industry. Once modified, the shrimp would tumble and mix within the rotating flask instead of sliding in a mass. The flask was attached to a rotary evaporator which functioned to provide variable rotate speed and degree of vacuum.

With this simple unit, we were able to visually monitor the effects of the tumbler rotation speed (physical manipulation of the shrimp), angle of tumbler, vacuum versus non-vacuum, and the ratio of chemical solution/shrimp on all samples treated. This lab scale

RESULTS AND DISCUSSION

The results of all chemicals screened were graphed so that the influence on the shrimp sample could be visually followed through all processing stages (Figure 2). For example, the chemical SBS was used at increasing concentrations on 51-55 count (number of shrimp tails/pound) peeled and deveined (P&D) domestic brown shrimp. The percent change in moisture content at the different stages of processing is shown on the left vertical axis. The different stages of processing are shown on the horizontal axis. Influence of a specific concentration of SBS on a shrimp sample can be followed from the initial sample weight, through treatment, freeze/thaw and cook stages (Figure 2).

As with most phosphates, figure 2 clearly shows that as the concentration of SBS increases, the sample weight following treatment increases as well. In this example, the final cook yield also increased with increasing concentration of SBS. In evaluating the total influence of this chemical on the shrimp the sample was organoleptically rated after cooking, as shown on the right side of the graph. The rating scale is described in Table 2. As the concentration of SBS increased, the organoleptic evaluation became more negative. Therefore, it is imperative that a chemicals influence on a seafood product be judged not only by its moisture change at different processing stages, but also be evaluated organoleptically.

Table 2. Rating Scale (1-5)

1. Texture of treated sample is same as control or positively affected.
2. Texture of treated sample is similar to control, with slight transparent effects.
3. Treated sample has pulay texture. Is moderately transparent. Cooked texture is moderately rubbery.
4. Treated sample has glassy appearance. Cooked sample is transparent to the extent that it appears uncooked. Rubbery texture.
5. Transporant and rubbery. An unacceptable product.

Certain processors may only be interested in the moisture or weight increase during treatment. However, this could lead to a false evaluation of the chemical, especially when further processing is planned. For example, FP15 had moisture uptakes of 10-35 percent during treatment (Figure 3). However, during he freeze/thaw cycle all of the weight gained during treatment was lost. After cooking there was more than 20 percent moisture loss from the initial weight of the sample. Also, the use of vacuum did not significantly increase final product yield. Therefore, by looking at the influence of the chemical at all stages of processing, the evaluation of he chemical is more valid.

Fig. 2 Standard Procedure

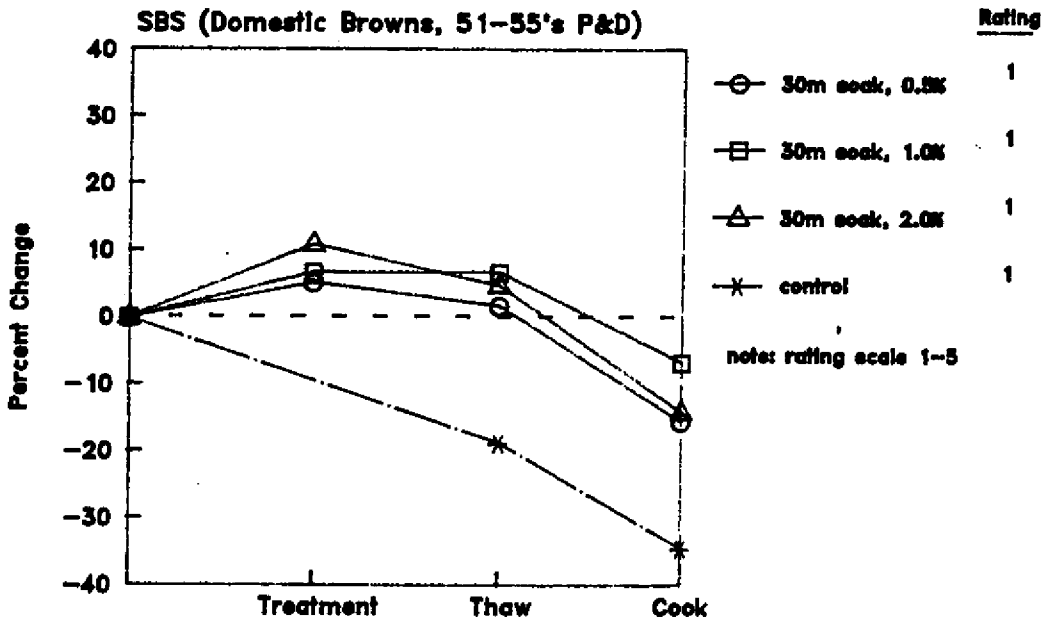


Fig. 3 Standard Procedure

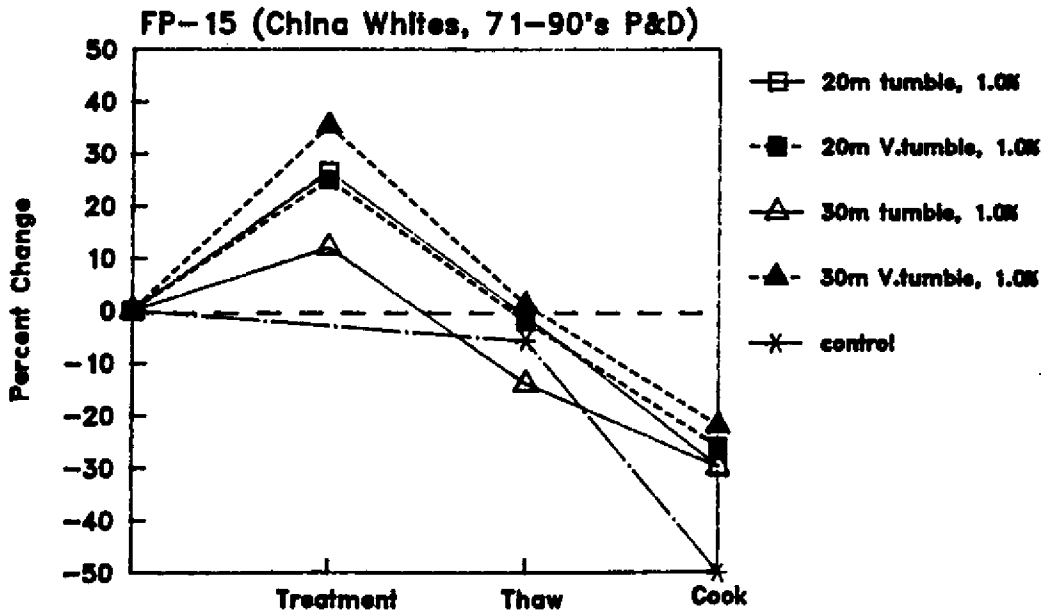


Fig. 4 Standard Procedure

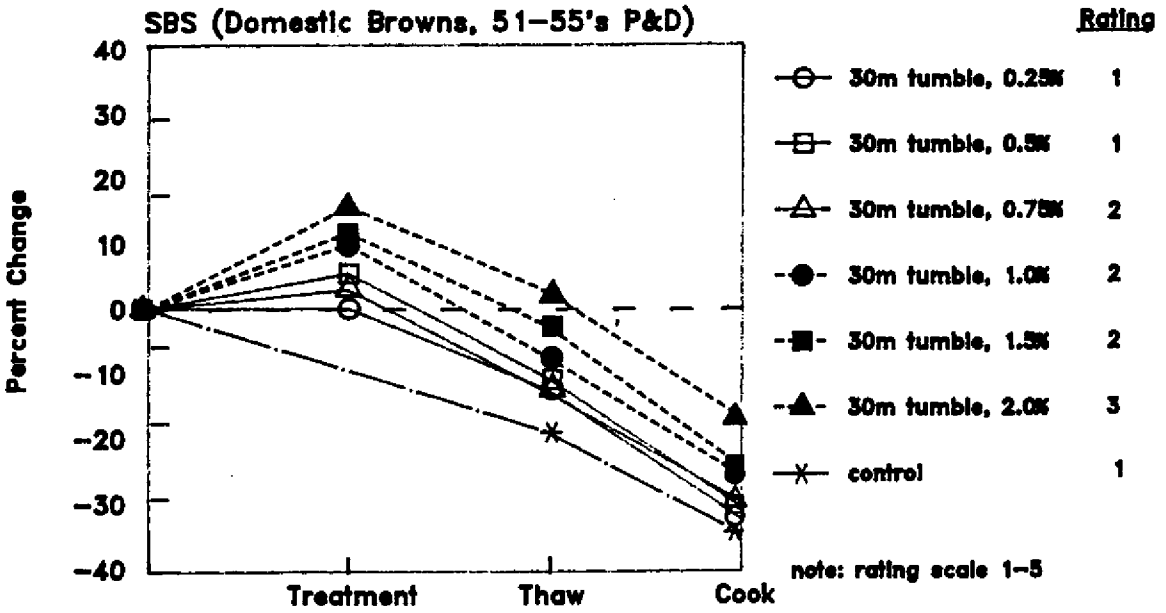
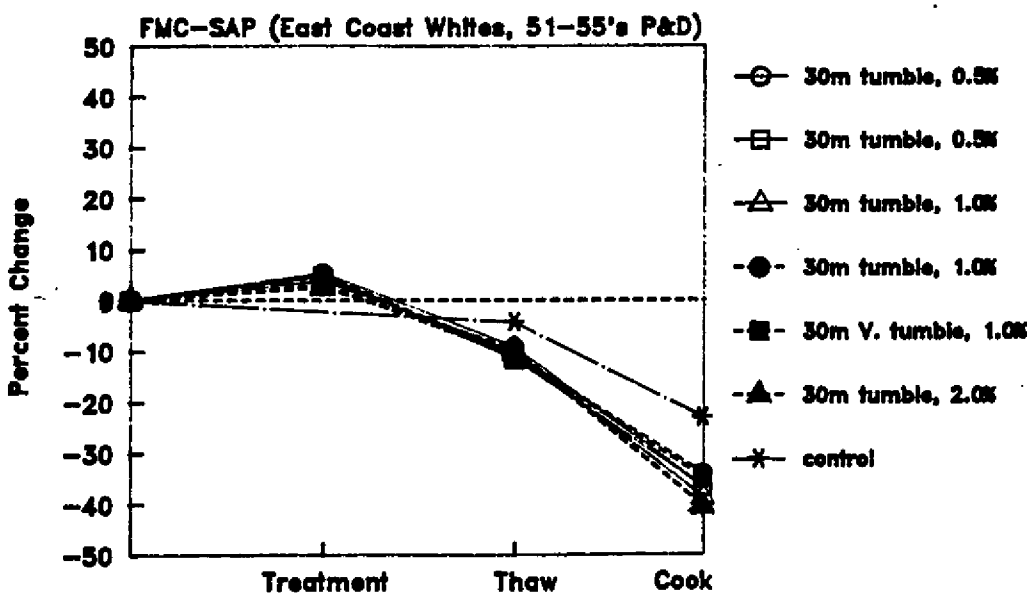


Fig. 5 Standard Procedure



In the previous two figures, the method of application of the chemical was tumbling. In figure 4, the method of application is soaking. In general, a shrimp sample will have lower moisture uptake from soaking then from tumbling in a given chemical solution. However, after examining the results in these few examples at all processing stages and the organoleptic rating of the treatments, an excellent overall rating is given to SBS when soaking is the method of application (Figure 4). SBS was the chemical used and illustrated in figures 2 and 4. With all factors the same, except method of application, SBS concentrations of 0.5, 1.0 and 2.0 percent have marked different results. There was less uptake during the soaking application but less freeze/thaw and total cooking losses as well. The overall organoleptic ratings for the soaked samples were much higher than those tumbled. Therefore, the processor must decide on the desired end-product when screening a chemical and its method of application.

Phosphates are probably used more than any other type of chemical additive for moisture control in seafood products. With all the different types of phosphates, there are as many different consequences. An example of a phosphate that resulted in little or no significant moisture uptake in a shrimp sample is seen with the chemical FMC-SAP (Figure 5). Not only is there no significant change from the initial weight following treatment, after freeze/thaw and cooking the sample weight is below the control sample weight. One reason a particular chemical may fail to have positive results for moisture control is that it may not migrate into the product.

The lab scale tumbler was used in conjunction with a commercial tumbler in several cases. The data from each of the tumblers followed the same trend for each specific chemical.

For most chemical treatments, tumbling yielded higher increased treatment weights than direct soaks. However, the data presented suggest that a chemical must be evaluated at all stages of processing. Tumbling with vacuum did not significantly increase the incorporation and extension of the chemical additive and moisture. It appears that he optimum tumble and soak time for moisture uptake and retention is 30 minutes.

The mini scale tumbler offers simple methodology on a small scale for controlled applications. It also offers a rapid way to predict consequence of additives that are used for moisture control. This mini tumbler offers a simple way to screen for validity due to inherent differences in raw materials, ingredients, and physical application.

Quality Evaluation of Soft-Shell Crawfish as Effected by Post-Harvest Holding Conditions

by

E.D. Park[1], J.S. Godber[1], D.D. Culley[2], B.W. Walker-Bryan[2], and M.W. Moody[3]

[1]Department of Food Science
[2]Department of Wildlife and Fisheries
Louisiana Agricultural Experiment Station
LSU Agricultural Center
Baton Rouge, Louisiana

[3]Louisiana Cooperative Extension Service
Baton Rouge, Louisiana

INTRODUCTION

Crawfish, considered a delicacy in Louisiana for many years, is found abundantly in bayous around the State. Although the major source of this product has been through harvesting in the natural environment, this species has become an important aquacultural crop for the region. Traditionally, only hard-shell crawfish have been readily available to the consumer. Soft-shell crawfish also have been consumed in Louisiana for many years, though it was not until the development of intensive culture systems in 1985 that soft-shell crawfish emerged as a commercial product (Culley et al., 1985). This high-priced gourmet product has since attracted the attention of many prominent Louisiana restaurants, being featured on some menus for the last several years (Barnett, 1988). For these reasons the soft-shell crawfish industry has rapidly expanded. Unfortunately, this rapid expansion of production has not been accompanied by the development of information on proper handling, processing and storage of this product.

One area of concern involves post-harvest holding conditions and times, and how these might affect the quality of soft crawfish. The current procedure used by producers involves removing freshly molted crawfish immediately from their trays

daily and placing them in refrigerated water at 2°C (post-harvest holding) until ready for packaging. This prevents hardening of the carapace. Crawfish are rarely left overnight without packaging due to the expectation for hardening of a certain portion of the crawfish beyond marketability. Packaging commonly consists of 450-680 g of crawfish in water per 1 liter plastic bag. This is then frozen, because fresh soft crawfish are easily damaged by handling (Culley & Duobinis-Gray, 1989).

The objective of this study was to determine the effects of different post-harvest holding solutions and durations on soft crawfish quality. The quality of soft crawfish was evaluated by sensory analysis, focusing on the characteristics of flavor intensity, juiciness, and muscle and exoskeleton texture. Other analyses included objective analyses of water dynamics, moisture content and lipid oxidation.

METHODS

Processing

Live soft crawfish (Procambarus clarkii) were obtained from two producers in the Baton Rouge, La. area. Approximately 3.8 Kg of crawfish were randomly placed, 2 crawfish deep, in each of 4 holding trays. Holding conditions were in 5 liters 1% NaCl solution (SALT); 5 liters distilled water (H_2O); 5 liters 0.5% solution tripolyphoshate (TPP) and Dry Storage on paper towels. All solutions and towels were changed daily. At 0(12 hrs), 2, 4 and 6 days, random samples were taken and placed in ziplock bags. Bags were filled with water, then mechanically frozen and stored at -20°C for one week in order to simulate commercial conditions (Culley & Duobinis-Gray, 1989). Samples from all treatments were analyzed following thawing in a water bath at room temperature.

Sensory Analyis

Nine experienced panelists assessed the product for flavor intensity, juiciness, initial (meat) texture and residual (exoskeleton) texture. Six training sessions were held prior to actual product evaluation. Terms and relative intensities were discussed with regard to product characteristics being evaluated. Samples were deep-fried in partially hydrogenated soybean oil at 375°F for approximately 1 min.

Samples were presented simultaneously with a fresh sample in a random fashion. Panelists were in partitioned booths under red fluorescent lighting. A 0.03% citric

acid solution and water were available for cleansing the palate between sample evaluations. A 100-mm unstructured horizontal line marked with verbal anchors of low and high intensity characteristics was used, yielding scores from 1 to 100 (Wang & Brown, 1983). Panelists were also asked to comment on the nature of flavor intensity. All samples were done in duplicate.

Water Dynamics

After thawing in a water bath at room temperature, crawfish were placed on absorbent paper toweling and allowed to drain for 15 minutes prior to weighing. Driploss (%) was calculated using the equation: [(packed weight-thawed weight) X (1/packed weight) X 100].

Moisture Content

Total moisture content was determined in duplicate by drying overnight at 102^{o}C to a constant weight (AOAC, 1984). Expressible moisture was determined in duplicate by the method of Jaurequi et al. (1981).

Lipid Oxidation

Lipid oxidation was determined using the 2-thiobarbituric acid method (TBA) of Tarladgis et al. (1960), with absorbance measured on a Gilford Response Spectrophotometer at 532 um. TBA numbers were determined using a standard curve of 2,2,3,3-tetramethoxypropane. The TBA values are the means of two replicates determined by the average of two distillates per sample.

Statistical Analysis

A randomized block design (RBD), blocking on panelists, was employed for sensory analysis. Objective analyses were analyzed using a completely randomized design (CRD). Data was analyzed using the General Linear Model (GLM) with the Statistical Analysis System (SAS, 1985). Differences between means were determined by least significant difference (LSD).

RESULTS AND DISCUSSIONS

Driploss for the four treatments, evaluated over time, are illustrated in Figure

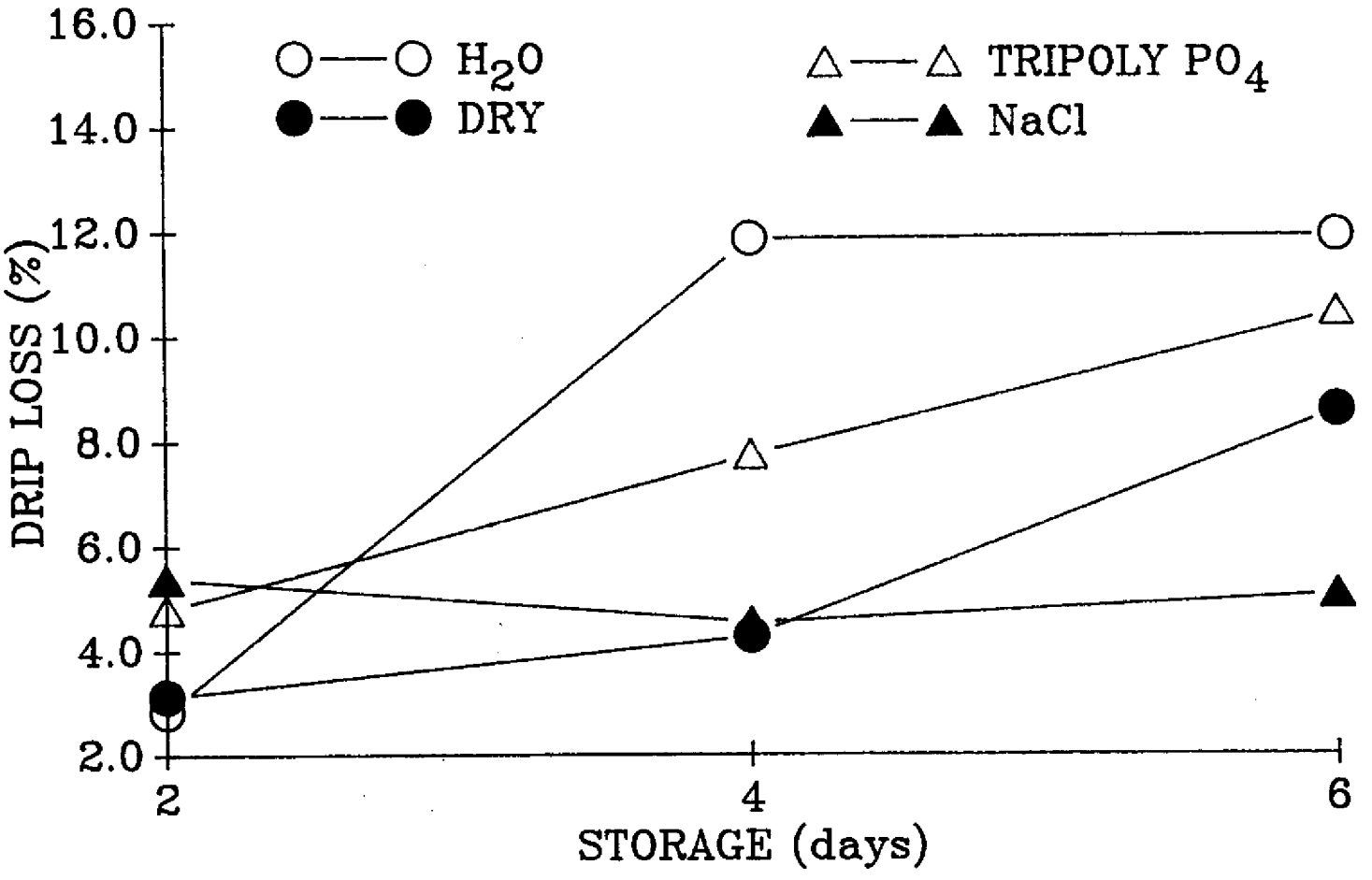


Figure 1. Influence of holding treatment and storage time on drip loss of soft-shell crawfish.

1. The measurement of drip is a well established indicator of quality in frozen seafoods (Shenowda, 1980). Furthermore, it correlates well as an indicator of protein denaturation (Jul, 1984). Samson et al. (1985) showed that freeze denaturation of proteins lowered their ability to retain water, thus increasing drip. Protein denaturation is often attributed to freezing and freezing rates, but can also result from chemical interactions with components such as lipid oxidation by-products (Sikorski, 1976). In either case, the result is a lower quality seafood product by making it drier and tougher. Current information indicates that driploss for soft crawfish is between 5 and 7 percent. Similar values were obtained in this study after two days of holding. However, after 4 and 6 days of holding, three of the treatments showed a marked increase in drip, with the H_20 treatment having values as high as 12 percent. In contrast, SALT remained fairly constant for the period examined. This result is surprising since low levels of salt usually result in protein denaturation and a corresponding loss of water.

A reduction of moisture content in the SALT treatment had indeed occurred, as illustrated in Figure 2. The moisture content of the SALT treatment was significantly lower ($p < .05$) than the H_20 and TPP treatments. It is possible that the SALT treatment caused a reduction in moisture content prior to packaging and that this in turn may have resulted in less driploss. This can not be confirmed in this study, however, since moisture content analysis was performed after packaging. It should also be noted that overall, moisture content on day 4 was significantly lower ($p < .05$) than on day 0 (12 hrs).

Water holding capacity, as indicated by expressible moisture, declined significantly ($p < .05$) after 2 days of holding (Figure 3). This decline was reversed however, after 4 and 6 days of holding. The H_20 treatment showed a lessened ability ($p < .05$) to retain water compared with the Dry Storage treatment. Overall, this analysis was not considered to be a useful indicator of changes during post-harvest holding due to the somewhat erratic results.

Another source of quality deterioration is lipid oxidation. This not only effects flavor quality of a product but has been suggested to contribute to textural deterioration or toughening of proteins in foods by forming insoluble aggregates (Karel, 1973). In all instances, levels of oxidation were considered to be low, with TBA values below 1 (Figure 4). These low levels could also indicate interactions with the protein component of the crawfish (Castell, 1971). However, the TPP treatment had appreciably higher ($p < .01$) TBA values than all other treatments. The SALT ($p < .01$) and H_20 ($p < .05$) treatments had the lowest levels of oxidation compared with other treatments but were not different from each other.

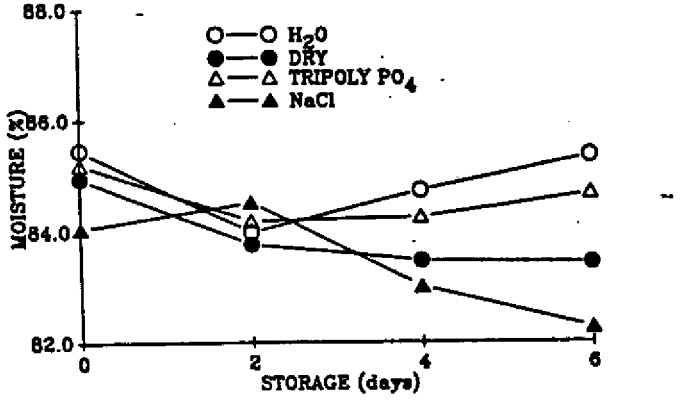


Figure 2. Influence of holding treatment and storage time on total moisture content of soft-shell crawfish.

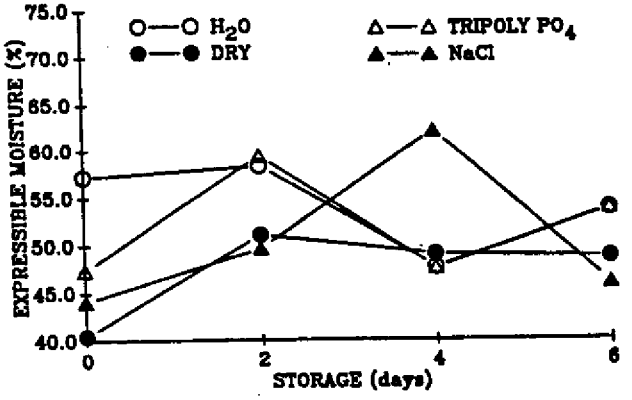


Figure 3. Influence of holding treatment and storage time on expressible moisture of soft-shell crawfish.

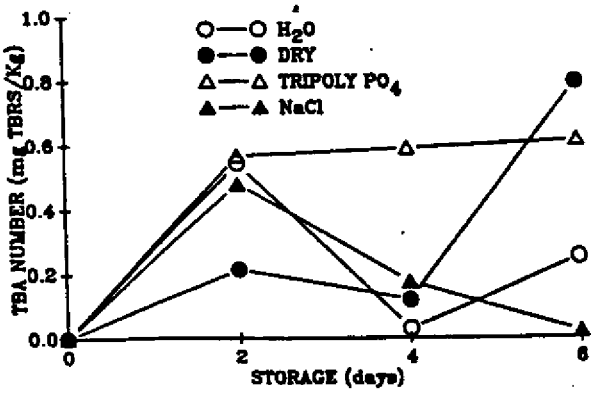


Figure 4. Influence of holding treatment and storage time on oxidation of soft-shell crawfish.

Higher levels ($p < .01$) of oxidation were observed for all storage times than at day 0 (12 hrs) with the highest levels ($p < .01$) occurring at days 2 and 6. This would indicate that oxidation took place during the post-harvest holding time rather than the 1 week of frozen storage.

Sensory analysis of flavor indicated that fresh samples, given as a control throughout the study, received an average score of 61 on flavor intensity (Table 1). SALT and Dry Storage treatments were higher ($P < .05$) than the fresh control. Panelists were also asked to comment on the nature of the flavor intensity. An average of 10 percent of the fresh samples were noted as having an off-flavor. The SALT treatment showed the lowest incidence of off-flavor, ie. 20 %, among the treatments examined, while Dry Storage had the highest with 40 %. Both H_2O and TPP had an average 33 % of samples with a disagreeable off-flavor.

Table 1. The Effect of Post-Harvest Holding Conditions on Flavor Intensity as Determined by Sensory Analysis

Method	Holding Time (Days)			
	0*	2	4	6
Fresh	$_{e}63.4^{a}$	$_{e}57.3^{a}$	$_{e}63.2^{a}$	$_{e}59.2^{a}$
Salt	$_{e}64.7^{a}$	$_{e}68.0^{ab}$	$_{f}73.6^{b}$	$_{f}71.0^{ab}$
Water	$_{e}61.6^{a}$	$_{e}63.3^{a}$	$_{e}63.2^{a}$	$_{f}68.0^{a}$
TPP	$_{e}57.5^{a}$	$_{e}64.3^{a}$	$_{eg}64.5^{a}$	$_{e}58.4^{a}$
Dry Storage	$_{e}61.8^{a}$	$_{e}66.9^{a}$	$_{fg}68.8^{a}$	**

*; 12 hour
**; Not determined due to observed condition of spoilage
a,b; Means (n=9) in same row with same superscript are not different ($p<0.05$)
e,f,g; Means (n=9) in same column with same subscript are not different ($p<0.05$)

The juiciness of a seafood contributes greatly to the perceived sensorial quality of that product (Connell, 1975). Table 2 reveals that the fresh control was perceived as being juicier ($p < .05$) than all treatments. This would indicate that the freezing of soft crawfish, as is true in most seafoods, lowers moisture retention (Samson et al., 1985). SALT and Dry Storage were rated ($p < .05$) as being the juiciest among the treatments evaluated after 4 days of holding. This would seem

to contradict earlier objective data where these treatments had the lowest moisture contents. One explanation for this could be related to the difficulty that panelists had distinguishing between juiciness due to cooking oil uptake and that from the crawfish itself. The reduced moisture content of these treatments may have resulted in increased oil uptake, thereby giving the apparent inverse relationship between moisture content and juiciness.

Table 2. The Effect of Post-Harvest Holding Conditions on Juiciness as Determined by Sensory Analysis

Method	Holding Time (Days)			
	0*	2	4	6
Fresh	$_{e}72.2^{a}$	$_{e}66.9^{a}$	$_{e}73.6^{a}$	$_{e}68.9^{a}$
Salt	$_{f}47.0^{ab}$	$_{fg}45.4^{a}$	$_{f}54.4^{ab}$	$_{f}55.6^{b}$
Water	$_{f}49.2^{a}$	$_{g}39.5^{b}$	$_{g}40.5^{b}$	$_{g}41.2^{ab}$
TPP	$_{f}45.3^{ab}$	$_{f}50.1^{a}$	$_{g}45.2^{ab}$	$_{g}40.5^{b}$
Dry Storage	$_{f}47.5^{a}$	$_{f}48.6^{a}$	$_{f}55.4^{a}$	**

*; 12 hours
**; Not determined due observed conditions of spoilage
a,b; Means (n=9) in same row with same superscript are not different ($p<0.05$)
e,f,g; Means (n=9) in same column with same subscript are not different ($p<0.05$)

In soft crawfish, exoskeleton texture is considered to be a very important sensory characteristic since a tough carapace lowers marketability (Culley and Duobinis-Gray, 1989). The fresh control was found to have a more tender carapace or residual texture ($p < .05$) by panelists when compared to the treatments (Table 3). The SALT treatment was found to have a softer carapace texture, ($p < .05$) than both TPP and H_2O after 6 days of holding. An apparent trend in softening of residual texture occurred with increased holding time ($p < .05$) in the SALT and Dry Storage treatments indicating a breakdown of the soft exoskeleton. In contrast, H_2O displayed an initial toughening ($p < .05$) of the exoskeleton after 2 days of holding, with subsequent softening after that. This could possibly be due to the longer survival of crawfish in this treatment, thus allowing the hardening of the exoskeleton initially.

Table 3. The Effect of Post-Harvest Holding Conditions on Residual Texture as Determined by Sensory Analysis

Method	Holding Time (Days)			
	0*	2	4	6
Fresh	$_{e}65.3^{a}$	$_{e}61.8^{a}$	$_{e}66.4^{a}$	$_{e}62.3^{a}$
Salt	$_{f}36.9^{a}$	$_{f}36.6^{a}$	$_{f}43.4^{ab}$	$_{f}48.0^{b}$
Water	$_{f}43.3^{a}$	$_{f}29.6^{b}$	$_{f}32.9^{b}$	$_{g}36.8^{ab}$
TPP	$_{f}34.9^{a}$	$_{f}36.6^{a}$	$_{f}36.8^{a}$	$_{h}32.5^{a}$
Dry Storage	$_{f}30.1^{a}$	$_{f}40.8^{b}$	$_{g}47.7^{b}$	**

*; 12 hours
**; Not determined due to observed spoilage conditions
a,b; Means (n=9) in same row with same superscript are not different ($p<0.05$)
e,f,g,h; Means (n=9) in same column with same subscript are not different ($p<0.05$)

Trends in initial or meat texture were similar to those of residual texture. A general toughening of texture resulted in all treatments with frozen storage ($p < .05$) when compared with the fresh control (Table 4). Toughening with freezing is common for many seafood products and likely occurs with soft crawfish as well (Sikorski et al., 1976). The TPP and H_2O treatments produced the toughest meat texture ($p < .05$) after 6 days while Dry Storage and Salt produced the softest ($p < .05$) after 4 and 6 days, respectively. Extended holding of crawfish softened meat texture ($p < .05$) in the SALT and Dry Storage treatments. This is probably caused by proteolytic hepatopancreatic enzymes. The presence of proteolytic enzymes, such as collagenase, in the hepatopancreatic tissue of other crustaceans, including fresh water prawns, have been reported to be responsible for texture softening during iced storage (Nip et al., 1985). Similar enzymes have been associated with the mushy texture exhibited by whole hard, crawfish even after short periods of iced storage (Godber et al., 1986). Marshall et al. (1987) have stressed the importance of proteolytic enzyme activity in maintaining textural quality of crawfish meat.

Table 4. The Effect of Post-Harvest Holding Conditions on Initial Texture as Determined by Sensory Analysis

Method	Holding Time (Days)			
	0*	2	4	6
Fresh	$_{e}70.5^{a}$	$_{e}66.3^{a}$	$_{e}69.1^{a}$	$_{e}66.8^{a}$
Salt	$_{f}37.7^{a}$	$_{f}42.5^{a}$	$_{f}44.4^{a}$	$_{f}50.9^{b}$
Water	$_{f}46.6^{a}$	$_{f}36.5^{b}$	$_{f}37.6^{ab}$	$_{g}39.4^{ab}$
TPP	$_{f}38.5^{a}$	$_{f}41.2^{a}$	$_{f}41.1^{a}$	$_{g}35.5^{a}$
Dry Storage	$_{f}36.5^{a}$	$_{f}46.0^{b}$	$_{g}51.8^{b}$	**

*; 12 hours
**; Not determined due to observed spoilage conditions
a,b; Means (n=9) in same row with same superscript are not different ($p<0.05$)
e,f,g; Means (n=9) in same column with same subscript are not different ($p<0.05$)

CONCLUSIONS

Indications from this study are that soft crawfish can be held after harvesting for up to 6 days under refrigerated conditions with the most satisfactory holding solution being a 1% NaCl solution. Ongoing analyses in this study include Instron texture analysis and crude hepatopancreatic enzyme activity. Hopefully these analyses will provide further insight into some of the problems facing the soft-shell crawfish industry. This study also established that freezing soft crawfish affects the sensorial quality of the product. Evaluation of freezing methods and packaging materials and their effect on long term frozen storage of soft-shell crawfish is in progress. These studies will help provide needed technical data and information on the handling and packaging for the rapidly growing soft-shell crawfish industry.

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FATTY ACIDS OF ADIPOSE TISSUE AND MUSCLE OF SWINE FED DIFFERENT FAT DIETS

Yao-wen Huang, Philip E. Koehler and Robert Seerley*
Department of Food Science and Technology
and
* Department of Animal Science
University of Georgia
Athens, Georgia

The consumption of saturated fatty acids has been shown to increase plasma LDL-cholesterol in man (Mattson and Grundy, 1985) and the increase of LDL-cholesterol has been correlated with coronary heart disease. Many consumers believe that the consumption of saturated fat products causes heart disease. Conversely, polyunsaturated fatty acids especially omega-3 (n-3) fatty acid EPA and DHA decrease the amount of plasma LDL-cholesterol (Mattson and Grundy, 1985). HDL-cholesterol is the only lipoprotein capable of removing cholesterol from the body. With the population of this country becoming more diet and health conscious, it is of considerable importance to the meat industry to produce red meat products that are perceived as being more healthful. Fish oils are rich in omega-3 fatty acids and have a hypotriglyceridemic effect due to an inhibition in hepatic glyceride synthesis. Therefore, the objective of this study was to incorporate fish oil into the diets to feed the swine to examine the effect on the fatty acid composition of pork.

MATERIALS AND METHODS

Feeding design: sixty four crossbred pigs were assigned to one of four treatments consisting a control corn-soybean meal diet (1) and three similar test diets that contained 2% fish oil and 3% poultry fat (2), 2% canola oil and 3% poultry fat (3), and 5% poultry fat (4). All test diets were iso-lysine (Table 1). All pigs were fed with grower feed until they reached 48 kg then test diets were fed. Feed and water were provided ad libitum. Two pens of eight pigs were fed each diet.

Tissue sampling: Immediately after slaughter, the longissimus dorsi muscle and overlaying subcutaneous fat at the 13th rib were collected. Tissue samples were vacuum packaged and then deep freezed until ready to use for fatty acids analysis.

Fatty acids analysis: Fat samples used for fatty acid analysis were extracted from homogenate of muscle portion and fat portion by AOAC methods (1984). The methyl esters were prepared according to AOAC methods (1984) with some modification. Brown cresol purple (0.1g/250 ml alcohol) was substituted for methyl red as an indicator to determine if the sample was acid free.

TABLE 1. COMPOSITION OF DIETS

Item	Diet* 1 Control	2 Fish oil/ Poultry fat	3 Canol oil/ Poultry fat	4 Poultry fat
Corn	82.76	76.75	76.75	76.75
Soybean meal	14.6	15.61	15.61	15.61
Poultry fat	-	3.00	3.00	5.00
Fish oil	-	2.00	-	-
Canola oil	-	-	2.00	-
Dicalcium phosphate	1.12	1.16	1.16	1.16
Limestone	0.80	0.77	0.77	0.77
Salt	0.40	0.40	0.40	0.40
Trace mineral mix	0.05	0.05	0.05	0.05
Vitamin mix	0.15	0.15	0.15	0.15
Lysine	0.04	0.04	0.04	0.04
Copper sulfate	0.05	0.05	0.05	0.05
Antibiotic**	0.02	0.02	0.02	0.02
	100.00	100.00	100.00	100.00

* Diets were formulated to contain 14% crude protein, 0.7% lysine, 0.6% Calcium and 0.5% Phosphorus.
** 44 mg of chlortetracycline per kg of diet.

Gas chromatographic determination of fatty acid methyl esters was accomplished using a Varian model 3700 unit (Varian Associates, Inc., Palo Alto, CA) equipped with a flame ionization detector and a Varian CD 111 integrator. The column temperature was programmed starting with an initial temperature of 70 C, held for 5 min., with an increasing program rate of 10 C/min., and a final temperature of 190 C, held for 25 min. The detector temperature was set at 250 C with an injection port temperature of 220 C. The carrier gas flow rate was 20 ml/min. Glass columns (180 cm x 0.64 cm x 0.02 cm) were packed with GP 5% DEGS-PS on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA). The fatty acid peaks were quantified using an omega fatty acid standard (Supelco, Inc., Bellefonte, PA) and were computed on a ug fatty acid/g sample basis or weight percent.

RESULTS AND DISCUSSION

Except for the pork samples from pigs fed fish oil and poultry fat diet (Diet 2), the female pigs had lower moisture contents than those of male pigs (Table 2). However, the fat content was inversely proportional to the moisture content. Pork samples of female pigs had higher fat level than that of male pigs.

TABLE 2. FAT AND MOISTURE CONTENTS OF PORK FROM PIGS FED DIFFERENT FAT DIETS

Item	Sex	Diet 1	Diet 2	Diet 3	Diet 4
Fat	male	3.4*	2.1	2.3	2.3
	female	4.7	4.4	3.2	3.6
Moisture	male	69.3	61.6	69.2	68.8
	female	64.3	61.6	61.8	57.2

* Means of four determinations

The fatty acid composition and ratio of n-3 to n-6 fatty acids of lipids used in the diets are listed in Table 3. The values were given as wet-weight percentages. The data showed that palmitic acid (C16:0) was the predominant saturated fatty acid in all three lipid sources. However, oleic acid (C18:1) was the major fatty acid in canola oil and poultry fat at levels of 51.3% and 35.1%, respectively. For polyunsaturated fatty acids, canola oil contained 17.2% of linolenic acid (C18:3) and 25.7% of linoleic acid (C18:2), while the fish oil contained 20.5% of eicosapentaenoic acid (C20:5, EPA) and 12.3% of docosahexaenoic

TABLE 3. FATTY ACID COMPOSITION OF LIPIDS USED IN THE DIETS

Fatty acid	Lipid Sources		
	Fish oil (for diet 2)	Canola oil (for diet 2)	Poultry fat (for diet 3)
Saturated			
14:0	7.8	0.1	0.9
16:0	15.7	5.2	21.5
18:0	4.4		8.4
22:0			0.7
Total saturated	27.9	5.3	31.5
Monoenoic			
16:1	11.4	0.5	9.7
18:1	16.2	51.3	35.1
Total monoenoic	27.6	51.8	44.8
Polyenoic (n-3)			
18:3	2.1	17.2	1.8
18:4	6.0		0.4
20:5	20.5		0.1
22:6	12.3		
Total (n-3)	40.9	17.2	2.3
Polyenoic (n-6)			
18:2	2.1	25.7	21.5
20:4	1.4		
Total (n-6)	3.5	25.7	21.5
Total polyenoic	44.4	42.9	23,8
U/P	2.58	17.85	2.17
P/S	1.59	8.08	0.75
n-3/n-6	11.69	0.67	0.11

TABLE 4. FATTY ACID COMPOSITION OF DIFFERENT DIETS

Fatty acid	Diets				
	Grower	Diet 1	Diet 2	Diet 3	Diet 4
Saturated					
14:0	0.7	0.2	2.9	0.8	0.8
16:0	16.0	13.0	20.2	13.3	19.2
18:0	7.6	3.1	1.1		2.9
22:0					0.7
24:0					0.3
Total saturated	24.3	16.3	24.2	14.1	23.9
Monoenoic					
16:1	1.3	0.8	8.6	7.2	7.0
18:1	35.5	32.0	15.2	36.3	35.2
22:1					0.1
Total monoenoic	36.8	32.8	23.8	43.5	42.3
Polyenoic (n-3)					
18:3	3.1	2.8	3.2	9.6	2.9
18:4			2.1		0.2
20:5			3.3	1.3	0.3
22:6			3.8	0.3	0.1
Total (n-3)	3.1	2.8	12.4	11.2	3.5
Polyenoic (n-6)					
18:2	35.5	48.1	35.9	30.0	30.5
20:4	0.3	0.1	1.0	1.5	
Total (n-6)	35.8	48.2	36.9	31.5	30.5
Total PUFA	38.9	51.0	49.3	42.7	34.0
U/S	3.12	5.14	3.02	6.09	3.19
P/S	1.60	3.13	2.04	3.03	1.42
n-3/n-6	0.09	0.06	0.34	0.36	0.12

TABLE 5. FATTY ACID COMPOSITION OF PORK FROM MALE PIGS FED DIFFERENT FAT DIETS

Fatty acid	Diet 1		Diet 2		Diet 3		Diet 4	
	Meat	Fat	Meat	Fat	Meat	Fat	Meat	Fat
Saturated								
14:0	2.1	1.9	1.6	1.5	1.5	1.4	1.8	1.4
16:0	26.1	24.7	23.4	21.2	24.4	23.5	24.0	23.3
18:0	14.7	20.0	12.1	12.2	12.6	14.2	12.9	13.8
22:0								0.3
24:0	0.3	0.3					0.5	
Total	43.2	45.9	37.1	34.9	38.5	39.1	38.2	38.8
Monoenoic								
16:1	6.5	3.1	6.0	4.3	5.6	2.9	6.8	3.8
18:1	37.8	34.1	39.2	34.1	39.4	39.7	35.8	38.7
22:1				0.3				
Total	44.3	37.2	45.2	38.7	45.0	42.6	42.6	42.5
Polyenoic (n-3)								
18:3	1.0	1.6	1.1	2.1	1.4	2.3	1.3	1.7
18:4				1.1				0.4
20:5			1.1	0.6				
22:6			0.8	1.0				
Total n-3	1.0	1.6	3.0	4.8	1.4	2.3	1.3	2.1
Polyenoic (n-6)								
18:2	10.0	14.4	12.6	20.7	13.3	15.6	13.9	16.2
20:4	1.3		2.1	0.8	1.6	0.4	3.0	0.3
Totaln-6	11.3	14.4	14.7	21.5	14.9	16.0	16.9	16.5
Total	12.3	16.0	17.7	26.3	16.3	18.3	18.2	18.6
U/S	1.60	1.16	1.70	1.86	1.59	1.56	1.59	1.57
P/S	0.28	0.35	1.46	2.16	1.29	1.29	0.48	0.48
n-3/n-6	0.09	0.11	0.20	0.22	0.09	0.14	0.08	0.13

TABLE 6. FATTY ACID COMPOSITION OF PORK FROM FEMALE PIGS FED DIFFERENT FAT DIETS

Fatty acid	Diet 1		Diet 2		Diet 3		Diet 4	
	Meat	Fat	Meat	Fat	Meat	Fat	Meat	Fat
Saturated								
14:0	2.3	1.5	2.0	1.5	1.9	1.3	2.2	1.3
16:0	23.3	22.9	22.6	21.6	24.3	22.5	23.5	21.9
18:0	13.7	14.7	13.5	14.6	12.7	13.2	13.2	13.1
22:0							2.7	0.4
24:0	0.4		0.2		0.3		0.4	
Total	39.7	39.1	38.3	37.7	39.2	37.0	42.0	36.7
Monoenoic								
16:1	7.5	3.3	6.4	4.1	7.0	4.1	6.5	4.0
18:1	38.8	36.9	37.6	34.8	38.8	38.9	38.5	38.6
22:1								
Total	46.3	40.2	44.0	38.9	45.8	43.0	45.0	42.6
Polyenoic (n-3)								
18:3	0.6	1.7	1.3	2.0	1.3	2.6	1.2	1.7
18:4				0.8				
20:5			1.5	1.3	0.1			
22:6			0.7	0.8				
Total n-3	0.6	1.7	3.5	4.9	1.4	2.6	1.2	1.7
Polyenoic (n-6)								
18:2	11.9	18.7	11.8	17.7	11.9	17.5	11.7	19.0
20:4	1.6	0.4	2.4	0.8	1.7	0.5		
Totaln-6	13.5	19.1	14.2	18.5	13.6	18.0	11.7	19.0
Total	14.1	20.8	17.7	23.4	15.0	20.6	12.9	20.7
U/S	1.52	1.55	1.61	1.65	1.55	1.70	1.38	1.72
P/S	0.35	0.53	0.46	0.62	0.38	0.56	0.31	0.56
n-3/n-6	0.04	0.09	0.25	0.26	0.10	0.14	0.10	0.09

acid (C22:6, DHA). Poultry fat contained only linoleic acid (C18:2) and very low level of n-3 fatty acids. The fatty acid composition of fish oil is similar to that of Kifer et al. (1971).

The addition of lipids affected the fatty acid composition of tested diets (Table 4). The fatty acid profile of Diet 4 was similar to that of the Grower feed. The ratio of n-3/n-6 PUFA in Diet 2 and 3 showed a 3-6 times higher than all other diets. However, Diet 2 had higher contents of EPA and DHA and Diet 3 contained higher level of linolenic acid (C18:3). This was due to the different fatty acid composition of fish oil and canola oil. The fatty acids in Diet 3 is in agreement with the report of Kifer et al. (1971).

For the test pork, the data showed that palmitic acid was the predominant saturated fatty acid in all eight samples, averaging 56-63% of saturated FAME (Table 5 and 6). Oleic acid was the major monoethylenic fatty acid. Not surprisingly that only the pork samples from pigs fed with fish oil diet (Diet 2) contained EPA and DHA. The distribution of polyunsaturated fatty acids is in agreement with that of Kifer et al. (1971). The ratios of n-3/n-6 of samples of Diet 2 were highest among all samples regarding the sex of digs.

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AN ECONOMIC ANALYSIS OF POTENTIAL REGULATORY CHANGES IN THE MULLET AND SEATROUT FISHERIES IN FLORIDA

by Charles M. Adams, Robert L. Degner and Susan D. Moss[1]

INTRODUCTION

The stimuli for this research lie in proposed public resource management policy changes involving the finfish resources which provide the resource base for the small-scale, near-shore finfisheries of Florida. When this project was initiated and funded by the Marine Fisheries Initiative Program (MARFIN), the primary finfish species produced by these fisheries were mullet, spotted seatrout, and red drum. However, during the early stages of the research, the Florida Marine Fisheries Commission imposed a series of resource management regulations on the harvest of red drum, which precluded detailed analyses of the economic impact of such regulations on individual commercial fishermen. Thus, attention was turned exclusively to the two remaining major species.

Public resource management regulations, such as production quotas or the establishment of seasonal restrictions, should be guided by an understanding of the effect any regulation will have on public welfare. Ideally, the criterion by which regulations are adopted should eventually result in a net increase in public welfare. Without an understanding of the potential impacts of a public policy action, policy makers have little basis for a decision. The goal of this project was to provide some understanding of the potential direct, or "first-round," economic impacts of regulations involving the mullet and seatrout segments of the near-shore commercial finfish resources of Florida.

OBJECTIVES

Understanding the impacts of policy changes requires a knowledge of the existing industry structure. Since the mullet fishery is the major in-shore fishery in terms of value and volume of landings, analysis of the in-shore finfish industry focuses on the mullet subsector of the industry, with other species being addressed through their relation to the mullet fishery. Thus, the overall objective of this research was to determine the current economic status of Florida's mullet fishery and to explore the economic interrelationships mullet shares with other estuarine species in the commercial seafood production and marketing system. This major objective was addressed through the following specific objectives:

1. Describe long-term and seasonal production and market trends by counties or regions and product form for mullet and complementary species in Florida.

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[1]Robert L. Degner, Charles M. Adams and Susan D. Moss are Professor, Associate Professor and Economic Analyst, respectively, of the Food and Resource Economics Department, University of Florida.

2. Assess elements of industry structure, conduct, and performance within the Florida mullet production and marketing system as follows:
   a. Estimate annual boat-level earnings profiles for the production of mullet, seatrout and other complementary species, and impacts of alternative resource management policies, by county.
   b. Identify the basic elements of concentration, market share, integration, entry and exit patterns, product diversification, and marketing strategies which characterize the industry. These elements will be analyzed by species, product form and geographic region.
   c. Define market channels for the various mullet product forms on a volume and value basis as product movement occurs between producer, intermediate marketing firms, retail outlets, and export market.

For the sake of brevity, this report will only briefly discuss findings associated with Objectives 1, 2a, and 2c on a statewide basis. These findings provide the primary basis for the policy implications discussed. For a more detailed discussion of the findings of each objective, refer to the complete MARFIN research report (Degner, Adams, and Moss ;1989).

METHODOLOGY

To meet the study's primary objective, a descriptive analysis of market structure and conduct was performed. Briefly, the analysis entails a description of fishermen, primary handlers/processors (dockside fishhouses) and secondary handlers/processors (wholesalers). The flow of product through these subsectors is described in sufficient detail to understand what actions are performed on the product in each subsector or market level, and what portion of the product is routed or "channeled" to the various final markets.

The information required for descriptions and analyses came primarily from three sources: (1) time series data on landings and dockside values from the National Marine Fisheries Service (NMFS), (2) Trip Ticket data from the Florida Department of Natural Resources (FDNR), and (3) data gathered from two surveys conducted by project personnel.

NMFS time series data on landings and value were used to describe production trends at the fishing firm level and to describe the complementarity between species in the production stage. Trip Ticket data from FDNR were used to establish annual boat-level earnings profiles, which are also helpful in understanding the producing sector's dependence on various species.

The major source of information for describing market channels came from two surveys conducted by project personnel. The first concentrated on the primary handlers of mullet (fishhouses), while the second survey focused on secondary handlers (wholesalers). Primary and secondary handlers of mullet were targeted because of their knowledge of production and marketing systems.

INDUSTRY DESCRIPTION

Background

Florida provides a major portion of U.S. landings of black mullet (85 percent) and spotted seatrout (62 percent). Mullet contributed ten percent of Florida's total finfish landings value, while seatrout accounted for about three percent. The mullet fishery provides flesh for human consumption in fresh, frozen,salted, or smoked forms. During the roe season, red and white mullet roe are produced for human consumption, primarily for export to theOrient, while most carcasses split for roe are sold for use as crab bait. Seatrout is valued almost exclusively for its flesh for human consumption. Due to their importance to the state's finfish fishery, this study focuses on both the mullet and seatrout subsectors of the industry. Throughout most of the report, unless indicated otherwise, "mullet" refers to both black and silver mullet, and "seatrout" refers to the four most common species, i.e. spotted, gray, sand and silver seatrout. However, black mullet accounts for 98 percent of mullet landings and spotted seatrout account for 87 percent of seatrout landings.

Long-Term and Seasonal Production Trends

From 1977 through 1988, combined black mullet and spotted seatrout landings, on average, represented 24.4 percent of all finfish landed in Florida and contributed 14.6 percent of revenue earned by Florida's finfish industry (NMFS; FDNR). Black mullet landings, as a percent of Florida's total finfish landings, ranged from a high of 28.8 percent in 1978 to a low of 18.3 percent in 1987 and 1988. Black mullet revenue varied from a high of 17.1 percent of Florida finfish revenue in 1978 to a low of 8.3 percent in 1985. Spotted seatrout landings, as a percent of total finfish landings, varied from a high of 2.8 percent in 1979 to a low of 1.2 percent in 1988. Revenue earned from spotted seatrout landings ranged from 4.7 percent of total finfish revenue in 1978 to 1.9 percent in 1988. Simple linear regression analysis of landings and revenue data from 1977 through 1988 showed no significant change in the contribution of either black mullet or spotted seatrout to the total finfish catch of Florida. All prices and revenues are reported in 1986 dollars. All secondary price series have been adjusted by the Producer Price Index to reflect changes in the general price level.

Black Mullet Production and Revenue

Black mullet landings and revenue were somewhat variable during the 1977-88 period, with a downward trend in landings seen since 1981. In 1977, 21.6 million pounds of black mullet, with a dockside value of $6.7 million, were landed in the state of Florida (Table 1). By 1981, production had risen to a peak of 31.0 million pounds, with a dockside value of $7.5 million. After 1981, production began to decline and, by 1985, only 19.2 million pounds of black mullet, with a dockside value of $5.1 million, were landed. In 1986, landings again increased to 22.8 million pounds, with a dockside value of $7.4 million; but, by 1988, landings had dropped to 19.2 million pounds, valued at $6.7 million. The average price paid for

Table 1.--Annual landings, revenue and average prices for black mullet, spotted seatrout, other species and the total finfish industry, 1977-1988.

Year	Black mullet landings	Black mullet revenue	Average price per pound[a]	Spotted seatrout landings	Spotted seatrout revenue[a]	Average price per pound[a]	Other species landings	Other species revenue[a]	Average price per pound[a]	Total finfish landings	Total finfish revenue[a]	Average price per pound[a]
	(Pounds)[b]	(Dollars)[c]	(Cents)	(Pounds)[b]	(Dollars)[c]	(Cents)	(Pounds)[b]	(Dollars)[c]	(Cents)	(Pounds)[b]	(Dollars)[c]	(Cents)
1977	21.6	6.7	30.9	2.4	2.0	81.0	70.3	34.9	49.7	94.3	43.6	46.2
1978	26.5	7.5	28.3	2.4	2.1	85.5	63.2	34.2	54.2	92.2	43.8	47.6
1979	25.7	6.4	25.0	2.6	2.2	86.3	62.7	39.4	62.8	90.9	48.0	52.8
1980	29.6	6.9	23.3	2.5	2.0	79.3	86.1	45.8	53.1	118.3	54.7	46.2
1981	31.0	7.5	24.3	2.7	2.2	81.5	89.2	52.1	58.4	122.9	61.9	50.3
1982	26.4	6.1	23.1	2.7	2.5	90.3	90.9	60.1	66.1	120.1	68.7	57.2
1983	23.9	6.0	25.1	2.4	2.2	91.9	78.2	53.9	68.9	104.5	62.1	59.4
1984	19.8	5.1	25.7	1.9	1.7	90.9	79.0	50.3	63.7	100.7	57.1	56.7
1985	19.2	5.2	26.8	1.5	1.4	91.5	74.8	55.6	74.4	95.5	62.1	65.1
1986	22.8	7.4	32.6	1.6	1.5	97.7	93.4	58.7	62.8	117.8	67.7	57.4
1987	22.7	8.5	37.5	1.7	1.7	103.3	99.5	64.8	65.1	123.8	75.0	60.6
1988	19.2	6.7	35.1	1.3	1.1	86.4	84.1	50.3	59.8	104.6	58.2	55.6
Average	24.0	6.7	28.1	2.1	1.9	88.8	81.0	50.0	61.6	107.1	58.6	54.6

[a]All revenues and prices reported have been adjusted by the Producer Price Index to reflect 1986 dollars.

[b]Million pounds.

[c]Million dollars.

Source: Data for 1977-85 were obtained from the National Marine Fisheries Service unpublished state landings data. Data for 1986-88 were obtained from the Florida Department of Natural Resources, Division of Marine Resources unpublished Trip Ticket data.

black mullet in 1977 was about $0.31 per pound. By 1980, the price had fallen to $0.23. From 1981 to 1985, the dockside price received for a pound of black mullet gradually increased to $0.27, with the exception of a decrease to $0.23 in 1982. NMFS data from 1986 through 1988 showed mullet prices had increased to $0.33 in 1986, $0.38 in 1987, and $0.35 in 1988. The increased price level occurring after 1985 suggests a higher level of demand for black mullet, but annual data are insufficient to statistically analyze this occurrence.

The time of year that landings take place has a significant impact on the value of the black mullet catch. Beginning in the mid to late 1970s, the market for mullet roe began to increase in importance. Red roe is now a highly prized export commodity, the importance of which is shown by data from the primary handler survey. Surveyed fishhouses reported purchasing 44 percent of the annual mullet catch, representing 48 percent of estimated annual mullet revenue in 1986, during the three- to four-month "roe season" (Table 2). The surveyed fishhouses reported roe season begins earlier in northern counties of Florida--normally October, but as early as September. In southern counties, roe season does not begin until November but may last until February. This shifting roe season allows fishermen the opportunity to follow the roe mullet southward, thus taking advantage of an extended roe season. Landings of mullet in Florida varies considerably by county. The majority of the landings occur south of the Big Bend region of the state.

Spotted Seatrout Production and Revenue

Approximately 2.4 million pounds of spotted seatrout, with a dockside value of $2.0 million, were landed in the state of Florida in 1977 (Table 1). Spotted seatrout production reached a peak of 2.7 million pounds in both 1981 and 1982. The dockside value of spotted seatrout landings reached a peak of $2.5 million in 1982. After 1982, production began to decline and, by 1988, only 1.3 million pounds of spotted seatrout, valued at $1.1 million dockside, were landed. This represents a 44 percent decline from 1982 to 1988. The average price paid for spotted seatrout in 1977 was about $0.81 per pound. In 1979, the price rose to a high of $0.86 per pound but fell back to $0.79 per pound in 1980. After 1981, the price of spotted seatrout was consistently over $0.90 per pound, the price reaching a peak of $1.05 per pound in 1987. However, in 1988, the price dropped to $0.86 per pound.

Unlike black mullet, the time of year that landings take place does not significantly impact the value of spotted seatrout (NMFS; FDNR). However, like mullet, total spotted seatrout landings and revenue are highest in the winter months (November through January) and lowest in June and July. As for mullet, landings of spotted seatrout varies considerably across counties in Florida.

Fishermen Production and Revenue Profiles

The in-shore finfish fishery of Florida collectively targets a complement of species. Similarly, individual fishermen within the fishery also target a complement of species available for harvest from the near-shore waters. Prior to 1987, the major species of commercial importance were

Table 2.--Mullet landings and revenue in the roe season compared to non-roe season.

Season[a]	Landings[b]		Value[c]	
	(1,000 pounds)	(Percent)	(1,000 dollars)	(Percent)
Roe	15,646	44	4,312	48
Non-roe	19,531	56	4,746	52
Total	35,177	100	9,058	100

[a]Roe season, as reported by fishhouse operators included in the primary handler survey, ranged from September through December in the Florida Panhandle and Big Bend region of the gulf, and November through January in counties further south.

[b]Obtained from primary handler survey, 1986 data.

[c]Price estimates from NMFS data were used to calculate value.

Source: Primary handler survey.

mullet, red drum, seatrout, and a variety of other species of lesser total value. For reasons such as gear specialization or regional limitations, fishermen participating in this fishery may target all or some subset of these species. Additionally, the fishery is comprised of part-time as well as full-time fishermen. Therefore, the fishery is characterized by a heterogeneous production sector, in terms of species mix, volumes and value landed, full versus part-time involvement, and other factors, all of which vary on a regional basis.

Species Mix

Of the 4,937 fishermen that participated in the in-shore finfish fishery in 1987, 3,240 landed mullet, while 3,975 within the same total targeted seatrout (Table 3). Some fishermen targeted one species to the exclusion of the other. For example, 962 fishermen targeted mullet to the exclusion of seatrout, while 1,697 fishermen targeted seatrout to the exclusion of mullet. However, the majority of either mullet or seatrout were produced by fishermen who annually targeted a combination of the two species.

As such, the in-shore finfish fishery is characterized by species complementarity in production. For example, the majority of the landings were reported by fishermen who produced a combination of mullet, seatrout, and other species. Approximately 90 percent of the total landings were produced by 2,137 fishermen who produced a combination of mullet, seatrout and other species. These fishermen produced approximately 21.5 million pounds of mullet, 1.5 million pounds of seatrout, and 6.9 million pounds of other species. The remaining 10 percent of the landings were produced by the other 2,800 fishermen, who produced some subset of the three species groups.

The above discussion underscores the prevalence of complementarity in production among species targeted by the in-shore finfish fishery. This is especially true with regard to mullet and seatrout. Only seven percent of mullet were reportedly caught by fishermen who did not also target seatrout, while 18 percent of the seatrout were caught by fishermen who did not also target mullet. Fishermen who exclusively targeted mullet or seatrout represented only nine and 14 percent of the total number of fishermen, respectively.

Distribution of Fishermen Landings and Revenue

The ranges of annual landings of mullet and seatrout for individual fishermen were incrementally categorized. The resultant incremental listings indicate how many fishermen produce what volume of mullet and seatrout on an annual basis. Although the volume increments utilized were somewhat arbitrarily established, the landings distributions are informative. The following discussion describes these annual landings distributions for mullet and seatrout.

Mullet

The majority of the mullet landings can be attributed to a very small percentage of the total number of fishermen who targeted mullet. Those fishermen with annual mullet landings of between 1 and 29,999 pounds collectively represented 50 percent of the total mullet landings and revenues (Table 4). However, these categories contained 94 percent of the

Table 3.--Summary of landings and revenues for fishermen landing various combinations of species.

Species mix	Fishermen	Mullet		Seatrout		Other species		All species	
	(Number)	(Pounds)	(Dollars)	(Pounds)	(Dollars)	(Pounds)	(Dollars)	(Pounds)	(Dollars)
Mullet, seatrout and other species	2,137	21,469,452	5,614,542	1,534,868	1,519,519	6,919,336	2,352,574	29,923,656	9,486,635
Mullet and seatrout	141	142,557	37,505	14,337	14,194	0	0	156,894	51,699
Mullet	440	534,666	140,591	0	0	0	0	334,666	140,591
Seatrout	707	0	0	78,700	77,913	0	0	78,700	77,913
Mullet and other species	522	1,051,895	276,453	0	0	181,358	61,662	1,233,253	338,115
Seatrout and other species	990	0	0	284,566	281,720	786,557	267,429	1,071,123	549,149
All species	4,937	23,198,570	6,069,091	1,912,471	1,893,346	7,887,251	2,681,665	32,998,292	10,644,102

Source: Department of Natural Resources Trip Ticket data, 1987.

Table 4.--Distributions of fishermen's mullet landings.

Range of mullet pounds landed		Number of fishermen	Percent of fishermen	Cumulative percent of fishermen	Total mullet landings	Percent of mullet landings	Cumulative mullet landings	Cumulative percent of mullet landings
(-----Pounds-----)		(Number)	(Percent)	(Percent)	(Pounds)	(Percent)	(Pounds)	(Percent)
0	99	618	19.10	19.10	23,298	0.10	23,298	0.10
100	199	264	8.15	27.25	38,246	0.16	61,544	0.26
200	299	178	5.49	32.74	43,380	0.18	104,924	0.45
300	399	140	4.32	37.06	48,710	0.21	153,634	0.66
400	499	103	3.18	40.24	46,054	0.20	199,688	0.86
500	599	78	2.41	42.65	42,821	0.18	242,509	1.04
600	699	68	2.10	44.75	43,970	0.18	286,479	1.23
700	799	74	2.28	47.03	55,203	0.24	341,682	1.47
800	899	48	1.48	48.51	41,064	0.18	382,746	1.65
900	999	54	1.67	50.18	51,366	0.22	434,112	1.87
1,000	1,999	313	9.66	59.84	446,345	1.92	880,457	3.79
2,000	2,999	173	5.34	65.18	417,856	1.80	1,298,313	5.60
3,000	3,999	121	3.73	68.81	420,872	1.81	1,719,185	7.41
4,000	4,999	105	3.24	72.16	474,502	2.05	2,193,687	9.46
5,000	9,999	295	9.10	81.26	2,170,460	9.36	4,364,147	18.81
10,000	14,999	184	5.68	86.94	2,230,093	9.61	6,594,240	28.42
15,000	19,999	106	3.27	90.21	1,860,434	8.02	8,454,674	36.44
20,000	29,999	121	3.73	93.95	3,049,610	13.15	11,504,284	49.58
30,000	39,999	64	1.98	95.92	2,186,568	9.43	13,690,852	59.02
40,000	49,999	42	1.30	97.22	1,847,810	7.97	15,538,662	66.98
50,000	99,999	80	2.47	99.69	5,330,245	22.98	20,868,907	89.96
>100,000		10	0.31	100.00	2,329,863	10.04	23,198,570	100.00
Total		3,240	100.00		23,198,570	100.00		

Source: Department of Natural Resources Trip Ticket data for 1987 was used to determine total mullet landings.

Table 5.--Distributions of fishermen's seatrout landings and revenues.

Range of seatrout pounds landed		Number of fishermen	Percent of fishermen	Cumulative percent of fishermen	Total seatrout landings	Percent of total seatrout landings	Cumulative seatrout landings	Cumulative percent of seatrout landings	Total seatrout revenue	Percent of seatrout revenue	Cumulative seatrout revenue	Cumulative percent of revenue
(-----Pounds-----)		(Number)	(Percent)	(Percent)	(Pounds)	(Percent)	(Pounds)	(Percent)	(Dollars)	(Percent)	(Dollars)	(Percent)
0	99	2,200	57.36	57.36	84,310	3.36	84,310	3.36	83,000	3.36	83,000	3.36
100	199	438	11.02	68.38	82,584	3.27	126,894	8.63	81,950	3.27	125,620	8.84
200	299	233	5.86	74.24	56,814	2.26	183,508	8.90	56,046	2.88	181,674	8.60
300	399	152	3.82	78.06	52,882	2.77	236,390	12.36	52,253	2.77	234,017	12.36
400	499	116	2.92	80.98	51,580	2.78	287,980	15.06	51,074	2.78	285,101	15.06
500	599	96	2.42	83.40	52,573	2.75	340,553	17.80	52,047	2.75	337,148	17.81
600	699	71	1.78	85.18	45,873	2.40	386,426	20.20	45,414	2.40	382,562	20.21
700	799	64	1.61	86.78	47,330	2.47	433,756	22.66	46,857	2.47	429,419	22.68
800	899	45	1.13	87.93	38,281	2.00	472,037	24.66	37,898	2.00	467,317	24.68
900	999	36	0.90	88.81	37,034	1.94	509,071	26.62	36,664	1.94	503,981	26.62
1,000	1,999	225	5.66	94.37	314,357	16.44	823,428	43.05	311,213	16.44	815,194	43.06
2,000	2,999	81	2.04	96.41	193,248	10.10	1,016,676	53.16	191,316	10.10	1,006,510	53.16
3,000	3,999	52	1.31	97.71	180,059	9.41	1,196,735	62.57	178,258	9.41	1,184,768	62.56
4,000	4,999	34	0.86	98.52	105,557	5.52	1,302,292	68.09	104,501	5.52	1,289,270	68.09
5,000	9,999	42	1.06	99.57	265,505	14.03	1,567,867	82.03	262,726	14.03	1,551,996	82.02
10,000	14,999	10	0.25	99.83	115,332	6.03	1,703,219	89.06	114,170	6.03	1,666,167	88.06
15,000	19,999	3	0.08	99.90	48,185	2.57	1,752,404	91.63	48,003	2.57	1,734,841	91.63
>20,000		4	0.11	100.00	160,067	8.37	1,912,471	100.00	158,498	8.37	1,893,347	100.00
Total		3,975	100.00		1,912,471	100.00			1,893,347	100.00		

Source: Department of Natural Resources Trip Ticket data for 1987 was used to determine total seatrout landings. National Marine Fisheries Service annual landings and revenue data for 1981-1985 were used to determine revenue.

total number of fishermen who targeted mullet. The remaining 50 percent of the total mullet landings and revenues were attributed to only 196 fishermen, or six percent of the total. Fully one-third of the total mullet landings and revenues can be attributed to only 90 fishermen, or less than three percent of the total number of fishermen who targeted mullet.

Seatrout

As was found with mullet, the distribution of total seatrout landings and revenue is skewed toward categories of high production that are represented by only a few fishermen. Approximately 90 percent of the fishermen account for only one-fourth of the total seatrout production and revenues (Table 5). Alternatively, approximately 50 percent of the total landings and revenue of seatrout is produced by only four percent of the fishermen who targeted seatrout in 1987. One-third of the total landings and revenue from seatrout is generated by only one percent (59 individuals) of the total number of fishermen who reportedly targeted seatrout.

Market Channels for Whole Mullet and Mullet Products

Among the estuarine species examined by this study, the marketing system for mullet is by far the most complex and least understood. The complexity stems from the fact that mullet is not only harvested for its flesh, but is especially prized for its red (female) roe. Processing for roe results in large quantities of spent ("split") carcasses, some of which are further processed. Although most split carcasses are sold for bait, they are sometimes dressed or filleted, and some smoked or salted. Whole mullet and processed products frequently move through different marketing channels.

This section provides insights as to the relative importance of the various mullet products and to the prevailing market channels through which these products are distributed. Prior to entering the retail market, the distribution of mullet products is dominated by two major sectors - primary handlers, i.e. fishhouses, and the secondary handlers, i.e. processors and wholesalers. At first glance, the distinction between "primary" and "secondary" handlers may appear clear cut. In reality, many secondary handlers were also found to function as primary handlers of other seafood species, buying directly from fishermen. In addition, some primary handlers were found to be processing small volumes of mullet.

Primary Handlers

Primary handlers (fishhouses) surveyed indicated that they bought 34.6 million pounds of mullet in 1986 (Figure 1). Due to inadvertent error by surveyed fishhouses and untraceable double counting resulting from buying and selling to other fishhouses, it is likely that the estimated volume reported by fishhouses included in the survey is four to five million pounds too high. However, emphasis should be placed on the proportionate product flows through the market channels described below, rather than on absolute quantities. Fishhouses reported that about 70 percent of all mullet was obtained directly from fishermen, 13 percent landed with their own boats, and 15 percent from fishhouses that were not included in the

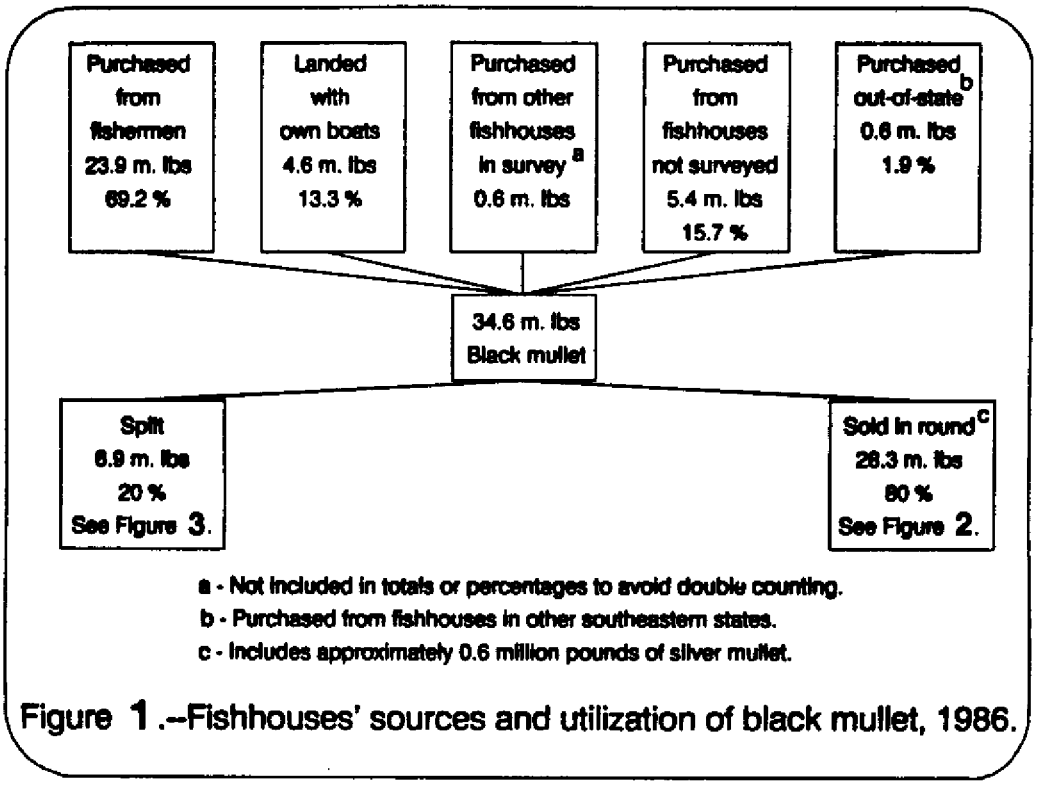

Figure 1.--Fishhouses' sources and utilization of black mullet, 1986.

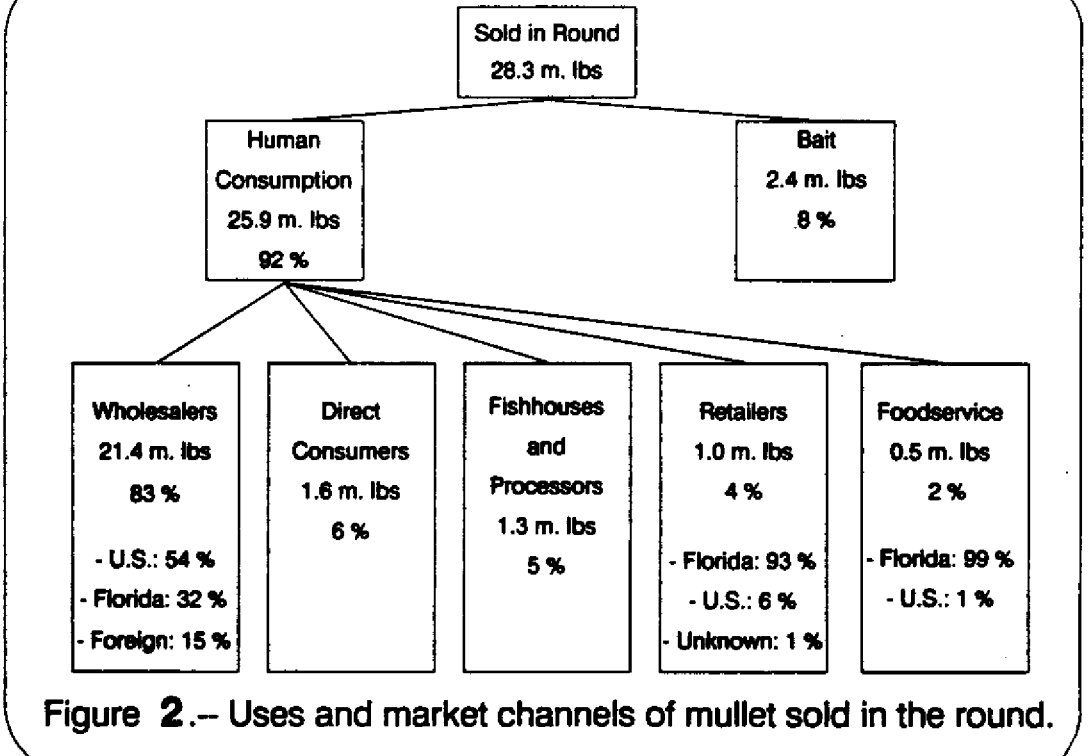

Figure 2.-- Uses and market channels of mullet sold in the round.

survey because they had gone out of business or refused to cooperate. About two percent of all mullet came from fishermen in adjoining states.

Mullet in the round

All primary handlers surveyed sold mullet in the round. In addition, these firms sold approximately 80 percent of their mullet volume in the round. This amounted to about 28.3 million pounds with a value of $9.9 million (35 cents per pound), representing about 59 percent of the total mullet value (Figure 2). About 70 percent was frozen and 30 percent fresh. Approximately 25.9 million pounds (92 percent) were sold for human consumption and 2.4 million pounds (eight percent) were sold directly to fishermen for bait.

Of the 25.9 million pounds of whole mullet going to human consumption, about 21.4 million pounds (83 percent) were sold to wholesalers. About 11.5 million pounds were sold to U.S. wholesalers outside of Florida (54 percent), and 6.8 million sold to Florida-based wholesalers (32 percent). Approximately 3.2 million pounds (15 percent) were sold to foreign wholesalers.

Fishhouses reported selling a total of 1.6 million pounds of mullet in the round directly to consumers. This represents about six percent of the total sold in the round for human consumption. Approximately 1.3 million pounds (five percent) were sold to other fishhouses or processors.

Sales to retail food stores and foodservice firms were relatively small. Total sales to food retailers amounted to slightly less than one million pounds, and nearly 93 percent of these were to Florida retailers. Foodservice firms bought about one-half million pounds, and virtually all were located in Florida.

Processed (split) mullet

Twenty percent of the annual landings (6.9 million pounds) were processed or "split," yielding high value roe and low value carcasses, although some split carcasses were processed into higher value products. The 6.9 million pounds of split mullet yielded products valued at nearly $7.0 million and accounted for about 41 percent of mullet's total annual wholesale value.

Processing for roe resulted in approximately 5.8 million pounds of split carcasses (including by-products), 1.1 million pounds of red roe, and about 33,000 pounds of white roe in 1986 (Figure 3). Florida fishhouses surveyed indicated that gizzards constituted an insignificant portion of their volume and revenue, and none was able to provide data on them.

Split carcasses.--About two-thirds of the fishhouses sold split mullet carcasses. About 91 percent of the resulting 5.3 million pounds of split carcasses and by-products were sold to fishermen for bait. In 1986, the average reported value was 15 cents per pound. A very small amount, 58,000 pounds (one percent), was used for pet food, and 462,000 pounds (eight percent) were used for human consumption (Figure 4).

Of the split carcasses used for human consumption, 71 percent were sold in a fresh or frozen form, while 23 percent were smoked and six percent salted. Although the total quantity of split carcasses used for human consumption was relatively small, the carcasses were marketed to a wide variety of outlets. Sales to secondary handlers (wholesalers) ac-

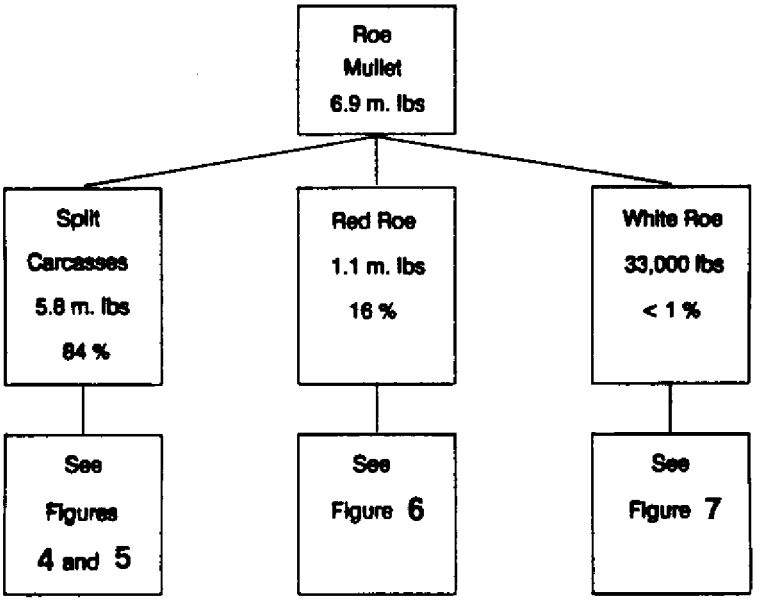

Figure 3.-- Products derived from roe mullet.

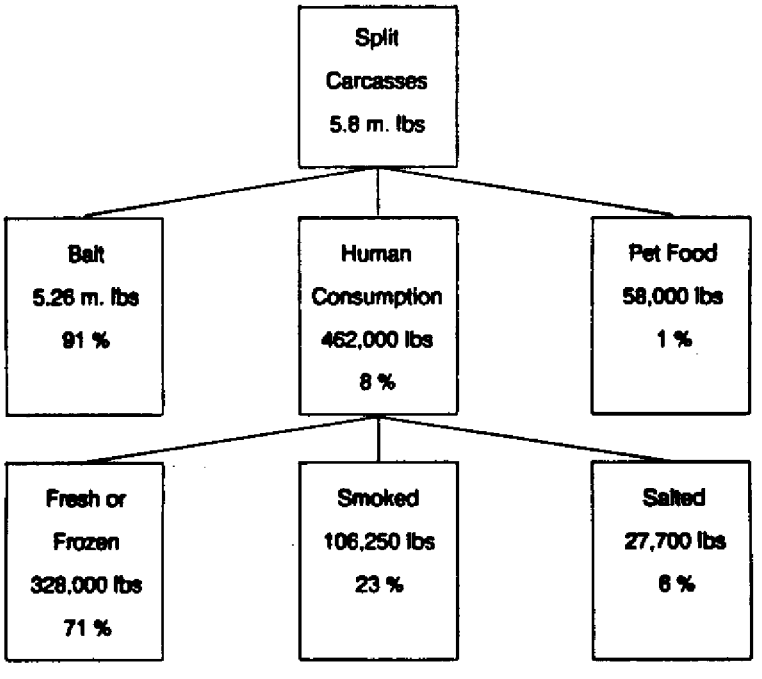

Figure 4.-- Product use and forms for split mullet carcasses.

counted for about 43 percent of the total, and the bulk of the volume was to Florida-based wholesalers (Figure 5).

About one-third of the split carcasses going for human consumption (152,000 pounds) were sold to foodservice outlets (restaurants), and almost all were located in Florida. Slightly over 55,000 pounds (12 percent) were sold directly to consumers through fishhouse-owned retail outlets, all of which were in Florida. Approximately 37,000 pounds (eight percent) were sold to other fishhouses in Florida and 18,500 pounds (four percent) to retailers, most of whom were thought to be located in Florida.

Red roe.--The fishhouses surveyed indicated that they collectively sold 1.3 million pounds of red roe, of which 1.1 million pounds (85 percent) were split on premises and 0.2 million pounds (15 percent) were presumably obtained from fishhouses not included in the survey (Figure 6).

As to distribution, about 1.25 million pounds (97 percent) were sold to wholesalers, 35,600 pounds to other fishhouses and processors in Florida, and extremely small quantities directly to consumers, to Florida retailers and to Florida restaurants. Of the 1.25 million pounds sold to wholesalers, two-thirds are sold directly to foreign firms, about one-fourth to Florida-based wholesalers, and about five percent to wholesalers in other states. The disposition of roughly three percent was uncertain. Even though significant proportions of red roe were sold to Florida-based wholesalers and wholesalers in other states, most fishhouse operators indicated that virtually all red roe was ultimately exported.

White roe.--White roe is a relatively minor product. The primary handlers reported selling only 33,000 pounds. Most white roe (46 percent) is sold directly to consumers by fishhouses through their own retail outlets. Thirty percent (10,000 pounds) is sold directly to Florida retailers. About 18 percent is sold to Florida wholesalers and only four percent sold to wholesalers in other states. An extremely small quantity reportedly went to other fishhouses in Florida (Figure 7).

Gizzards.--Not one of the primary handlers reported selling significant quantities of gizzards, although some of the secondary handlers indicated that gizzards were important to them. More aggressive marketing efforts could possibly increase revenues to Florida mullet processors.

Bait.--Mullet, both black and silver, are widely used for bait. Most firms, interviewed sold mullet for bait. Approximately 2.4 million pounds were sold in the round, and about 5.3 million pounds of split carcasses and by-products went to bait as well. Most is sold directly to fishermen by the fishhouses, but some is sold to other fishhouses for resale to fishermen.

Secondary Handlers

The primary handlers contacted were asked to identify "representative" wholesale seafood buyers (secondary handlers) of mullet. Fifty-eight firms were identified in this manner. Volume and sales data obtained were for 1987. Even though the sample of firms was not randomly selected, the high degree of cooperation and relatively large number of firms probably provides a reasonably accurate overview of secondary handlers' mullet marketing activities.

Although the secondary handlers in our survey were identified as such by firms in the primary handler survey, it is important to note that over

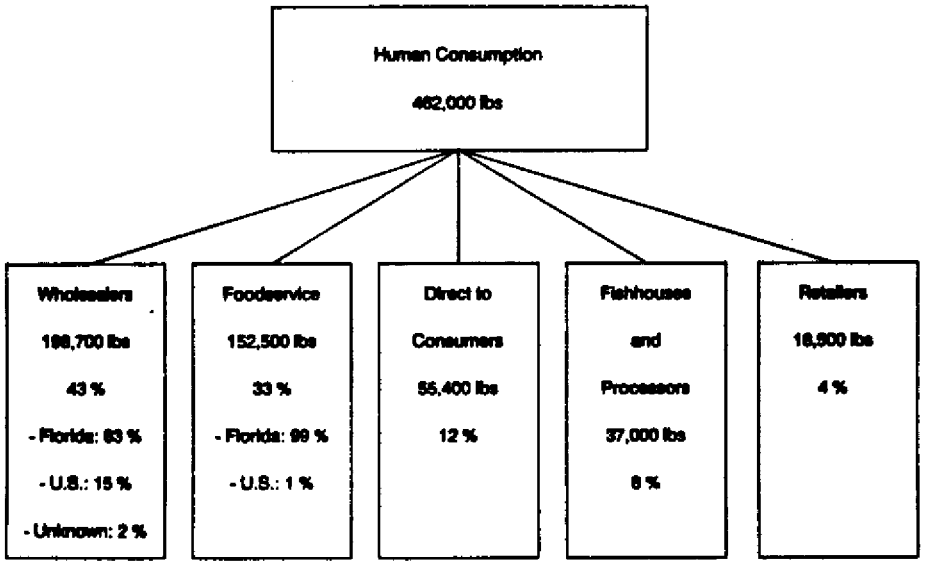

Figure 5.– Market channels for split mullet carcasses used for human consumption.

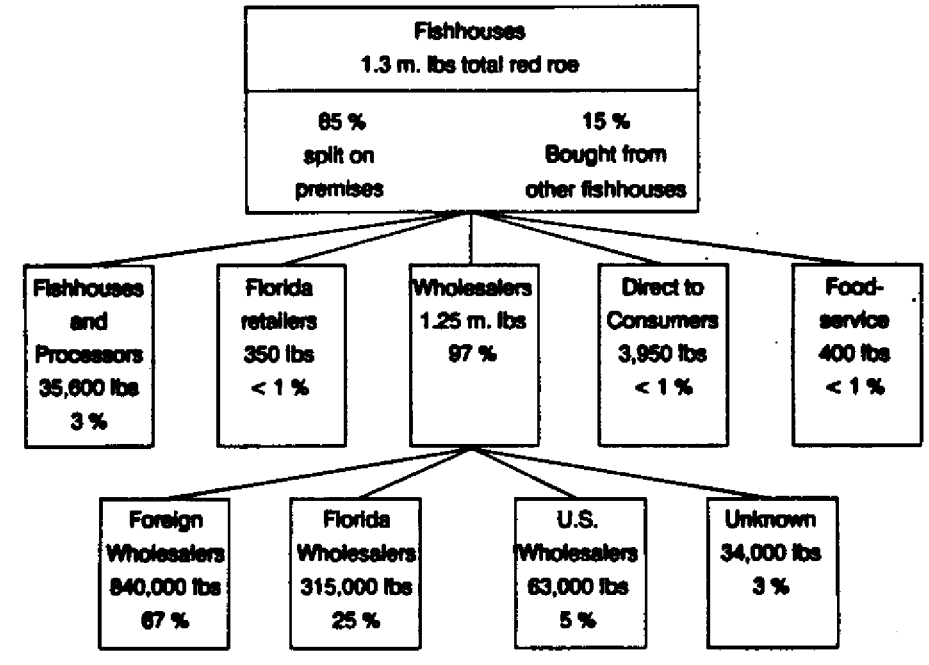

Figure 6.– Sources and market channels for red roe.

half of the secondary handlers indicated they also purchase seafood directly from fishermen. Thus, there is considerable "crossover" between our defined market levels.

Sources and distribution of whole mullet and mullet products

Mullet in the round.--The secondary handlers interviewed reportedly bought approximately 17.4 million pounds of whole mullet in 1987. About 14.5 million pounds (83 percent) originated in Florida, and 2.9 million pounds (17 percent) in other states (Figure 8). States mentioned most frequently as being significant suppliers of mullet in the round included Alabama and Louisiana.

Approximately 70 percent (12.2 million pounds) of the mullet acquired in the round are sold in the round, with the balance further processed before the next sale. Further processing includes "splitting" for red and white roe and gizzards (which also yields the spent or "split" carcasses), dressing and filleting, smoking, and salting. Obviously, some of the processing is done only during the roe season using "roe mullet," and some is done throughout the year.

Of the 12.2 million pounds sold in the round, the largest proportion went to U.S. retailers. Approximately 5.0 million pounds, accounting for 41 percent, were sold to food stores and retail fish markets (Table 6, Figure 8). Foreign wholesalers constituted the next largest market, taking 4.0 million pounds, a third of the mullet sold in the round. Fifteen percent (1.8 million pounds) moved to other U.S. wholesalers.

About seven percent of their mullet in the round were retailed to consumers. Relatively small quantities went to foodservice firms, with those being predominantly restaurants. Mullet entering the foodservice sector accounted for approximately 400,000 pounds, or three percent of the total sold in the round. Nearly 200,000 pounds, two percent, were sold to fishermen for bait (Figure 8).

Split carcasses.--Split carcasses were obtained either through processing for roe and gizzards or by purchasing from other firms. Approximately 56 percent of the split carcasses handled by secondary handlers (2.2 million pounds) were also processed by them, while 44 percent were purchased from other processors (Table 7). Florida, Alabama and Louisiana were the only states named as sources for split carcasses. Although some split carcasses are sold as dressed, filleted, smoked or salted product, 95 percent are simply sold as raw split carcasses. Of the 3.6 million pounds sold as raw split carcasses, approximately 95 percent are sold to fishermen for bait. Relatively small quantities are sold to other seafood wholesalers, retail stores, restaurants, and directly to consumers (Table 6).

Dressed or filleted.--About 80 percent of the 0.7 million pounds of dressed or filleted product was processed from mullet in the round. About 14 percent was obtained from split carcasses, and the remaining 6.2 percent (52,000 pounds) bought in the dressed or filleted form. Florida was the only reported source of this product form. About half of the dressed or filleted product was retailed directly to consumers by the secondary handlers. About one-third went to retail stores, and 13 percent to other wholesalers. Only four percent went to foodservice firms, and none was sold to foreign wholesalers.

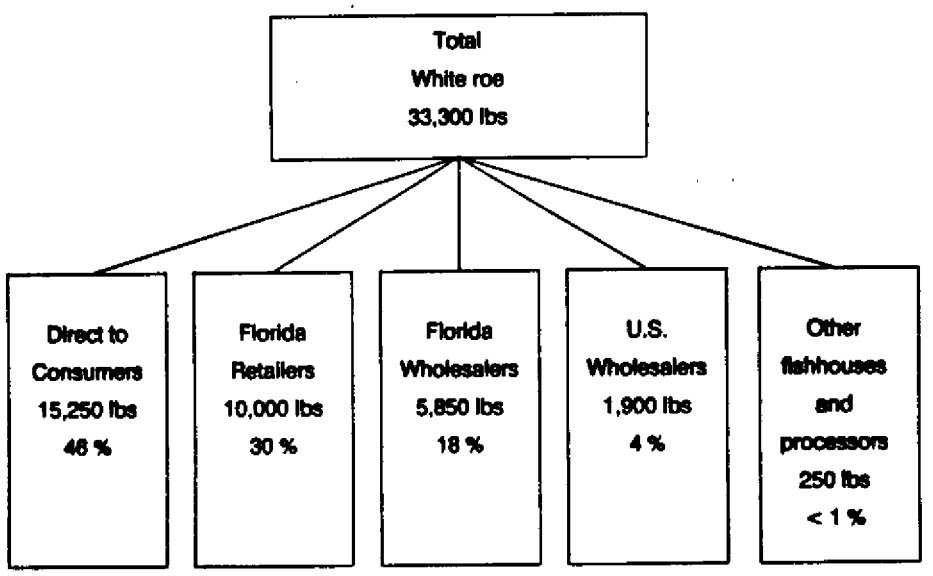

Figure 7.-- Market channels for white roe.

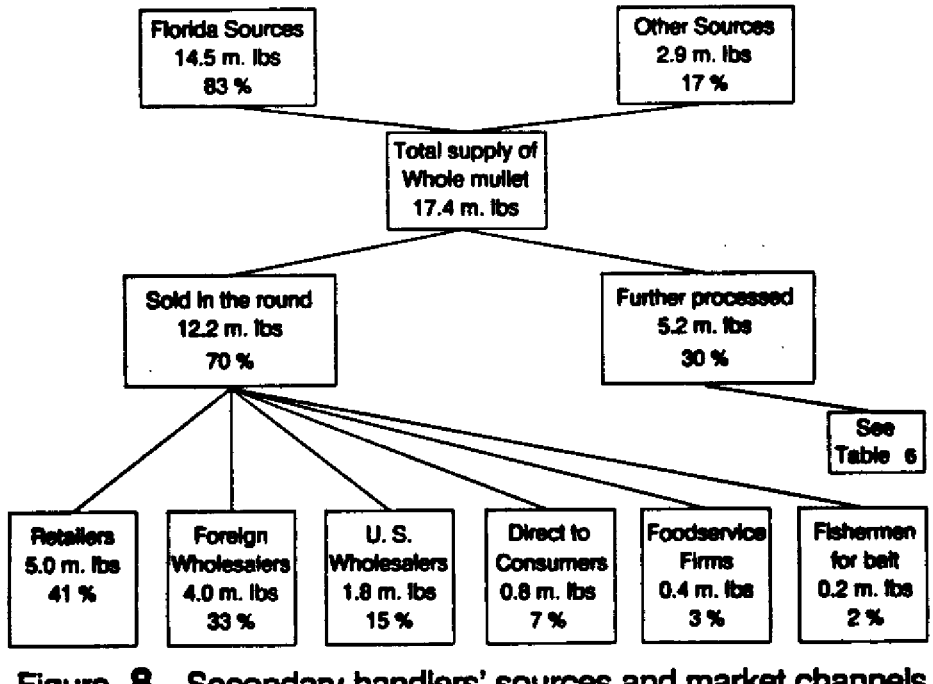

Figure 8.--Secondary handlers' sources and market channels for mullet in the round.

Table 6.--Distribution of selected mullet products by secondary handlers.

Market destination	Product							
	In the round		Red roe		White roe		Gizzards	
	(1,000 lbs.)	(%)	(1,000 lbs.)	(%)	(1,000 lbs.)	(%)	(1,000 lbs.)	(%)
Foreign wholesalers	3,982	33	1,832	99	20	32	156	100
U.S. wholesalers	1,779	15	3	--[a]	8	12	0	0
Retail stores	5,029	41	9	--[a]	26	40	--[b]	0
Foodservice firms	403	3	0	0	0	0	0	0
Direct to consumers	800	7	8	--[a]	10	16	1	--[a]
Fishermen for bait	198	2	0	0	0	0	0	0
Totals[c]	12,191	100	1,852	100	64	100	157	100

Market destination	Product							
	Split Carcasses		Dressed		Salted		Smoked	
	(1,000 lbs.)	(%)	(1,000 lbs.)	(%)	(1,000 lbs.)	(%)	(1,000 lbs.)	(%)
Foreign wholesalers	0	0	0	0	0	0	0	0
U.S. wholesalers	107	3	110	13	13	22	0	0
Retail stores	36	1	289	34	44	72	4	17
Foodservice firms	22	1	34	4	0	0	2	10
Direct to consumers	18	1	405	48	4	7	15	72
Fishermen for bait	3,464	95	0	0	0	0	0	0
Totals[c]	3,647[d]	100	838	100	61	100	21	100

[a]Less than one percent

[b]Less than 500 pounds.

[c]Totals may not be exact due to rounding.

[d]In addition to the split carcass totals shown here, approximately 164,000 pounds of split carcasses are dressed, 20,000 pounds are salted and 2,400 pounds are smoked. These quantities have been included in the appropriate product categories one a net yield basis, approximately 65,80 and 85 percent, respectively, for dressed, salted and smoked product.

Source: Secondary handler survey.

Table 7.--Sources and quantities of selected mullet products sold by secondary handlers.

Product	Quantities received/sold	
	(1,000 pounds)	(Percent)
Split Carcasses		
Split from whole mullet	2,165	56.4
Purchased	1,677	43.6
Subtotal	3,842	100.0
Split carcasses dressed	175	4.6
Split carcasses salted	18	0.5
Split carcasses smoked	2	0.1
Sold as split carcasses	3,647	95.0
Total split carcasses	3,842	100.0
Dressed or filleted		
Dressed from whole mullet	673	80.3
Dressed from split carcasses	113[a]	13.5
Purchased	52	6.2
Total dressed	838	100.0
Salted		
Processed from whole mullet	27	44.3
Processed from split carcasses	14[a]	23.0
Purchased	20	32.8
Total salted	61[b]	100.0
Smoked		
Processed from whole mullet	2	9.5
Processed from split carcasses	2	9.5
Purchased	17	81.0
Total	21	100.0
Red roe		
Split from whole mullet	526	28.4
Purchased	1,325	71.6
Total	1,852[b]	100.0
White roe		
Split from whole mullet	23	35.9
Purchased	41	64.1
Total	64	100.0
Gizzards		
Split from whole mullet	38	24.2
Purchased	119	75.8
Total	157	100.0

[a]Dressed salted and smoked yields are equal to 64.5, 80 and 85 percent of split carcass weights, respectively.

[b]Total does not agree due to rounding.

Source: Secondary handler survey.

Salted.--Two-thirds of the 61,000 pounds of the salted mullet handled by the secondary handlers was processed by them and one-third purchased from other processors located in Florida. Half of the total tonnage was processed from mullet in the round, and about one-fourth from split carcasses. Approximately three-quarters of the salted mullet were sold to retailers, and slightly over one-fifth to other wholesalers. Seven percent was sold directly to consumers, and none was reportedly sold to foodservice firms nor to foreign wholesalers.

Smoked.--The total quantity of smoked product handled was very small, only about 21,000 pounds. About ten percent was processed by secondary handlers from mullet in the round, and about 10 percent from split carcasses. Approximately 81 percent was bought already smoked from Florida firms. Secondary handlers sold nearly three-quarters directly to consumers, about 17 percent to retailers, and ten percent to restaurants. None went to other wholesalers, domestic or foreign.

Red roe.--Secondary handlers processed approximately 28 percent of the 1.8 million pounds of red roe handled, and bought the remaining 72 percent from processors in Florida, Alabama, Louisiana, Georgia and Mississippi, with Florida being the largest supplier. Virtually all is sold to foreign wholesalers. Very small quantities, about one percent of the total tonnage, is sold to other domestic wholesalers, retail outlets, and direct to consumers.

White roe.--About 64,000 pounds of white roe were reportedly marketed by secondary handlers. Roughly one-third was processed by them, and the remaining two-thirds purchased from processors in Florida, Louisiana, Alabama, Georgia and Mississippi. No state appeared to be particularly dominant in the white roe market. The relatively small quantities of white roe are marketed through a wide variety of outlets. The largest share, 40 percent, is sold to retail stores, an additional one-third is sold to foreign wholesalers, 16 percent directly to consumers, and 12 percent to U.S. wholesalers. None was reportedly sold to foodservice firms.

Gizzards.--Secondary handlers sold about 157,000 pounds of gizzards in 1987; about one-fourth were processed by these firms, and three-fourths bought from processors in Florida, Alabama, and Louisiana. Practically all were sold to foreign wholesalers. A fraction of one percent was sold directly to consumers, with none going to U.S. wholesalers, retail stores, or restaurants.

IMPACTS OF ALTERNATIVE RESOURCE MANAGEMENT POLICIES

The consequences of potential alternative resource management policies were assessed for mullet and seatrout. The specific policy alternatives chosen emerged from discussions and correspondence with the Florida Marine Fisheries Commission staff. The scenarios eventually used in the analysis reflect a set of possible management alternatives that were likely to receive attention by Commission staff, particularly for seatrout. In addition, other potential policy scenarios pertaining to mullet roe harvest were developed by the authors as a result of suggestions made by several primary handlers.

These policy scenarios are intended to offer benchmark measures for evaluating more specific policy options that may be developed in the

future. The potential policy measures were imposed on the mullet and seatrout fishery as currently managed. The consequences of each policy were measured in terms of changes in landings and total revenue as reported in 1987 Trip Ticket data. The policy alternatives were assessed by species and in combination among the two species. In addition, the impacts were assessed for all fishermen and for those fishermen who specifically target the species in question. These findings are reported on a county and state basis.

It is important to note that the impacts measured from these hypothetical policy alternatives should be considered as "first-round" effects. In other words, effects in the marketplace, such as price changes which may eventually occur as landings (and, thus, volumes available for sale in the market) change, were not estimated. In addition, the ability of fishermen to adjust effort among species has also not been estimated. For a complete assessment of the economic consequences resulting from imposing a given fishery management policy, these "second-round" effects in the marketplace may need to be measured.

Policy Descriptions

Several policy options were assessed for mullet and seatrout. The data utilized for this analysis were the Florida DNR Trip Ticket data for 1987. Landings for all mullet (black and silver) and all seatrout (spotted, gray, sand, and silver) were included and NMFS estimates of dockside prices for each species for 1987 were used. For mullet and seatrout, prices were weighted by volumes of the respective species. The policy alternatives for each species are discussed below.

Mullet

Three policy alternatives were assessed for mullet. The purpose of these options was to measure the impact on landings and revenue resulting from restrictions in the harvest of roe mullet. These three policy alternatives are listed as follows.

Option 1

Option 1 represents total elimination of mullet landings during the mullet roe season. This option eliminates mullet production during those months identified as the primary roe season months for each county. The months involved vary by county. The range of months representing the roe season for each county was established by the survey of primary handlers of mullet and further corroborated by NMFS landings data. Under this scenario, fishermen are allowed to harvest mullet only during the non-roe season, although production of complementary species during the roe season is permitted.

Option 2

Option 2 is a quota which limits the landings of roe mullet during each month of roe season to a volume equivalent to the average monthly mullet landings in the three-month period immediately preceding the roe season. The monthly quota is established independently for each county. In the examples calculated here, monthly distributions of mullet landings were calculated for each county for each month based on actual landings during 1981 through 1985. The resulting monthly percentages were then

applied to actual 1987 landings for each county. In practice, the average monthly quota could be based on the entire non-roe season or any other logical or equitable basis. The rationale for this option is to allow for landings that are sufficient to supply the flesh market.

Option 3

Option 3 reduced the roe mullet production by 50 percent from what would have been realized in the base year. For the purposes of this study, the "base" year was 1987, which was actually eight percent below the annual average for the preceding 10 years.

Seatrout

The policy options offered for seatrout were straightforward. The options examined essentially established annual production quotas for each county. These included a range of percentage reductions in annual seatrout landings from those landings reported in a base year, which was 1987. Reductions range from 10 to 50 percent, with incremental reductions of 10 percent. For example, Option 1 for seatrout would provide for fishermen to be able to land only 90 percent of the landings reported during the base year. Option 2 would provide for seatrout landings to be limited to 80 percent of the landings reported during the base year. Option 5 would represent a 50 percent reduction in seatrout landings as compared to the total annual landings of the base year. No monthly or trip quotas were examined.

Economic Impact on Fishermen

The economic impact resulting from imposing the various policy options is expressed in terms of changes in landings and total revenue. These economic consequences are discussed for both mullet and seatrout in Florida. The study examined the effects of imposing each policy, as well as policy combinations, on the total population of mullet and seatrout fishermen in each county and on a statewide basis. As one might expect, the counties more dependent on mullet and seatrout production exhibited the largest impacts as a result of the various policy combinations. However, this additional information is useful in assessing how commercial finfish landings and income in each county may be affected by policies jointly oriented toward the two most important remaining species targeted by the in-shore finfish fishery in Florida.

When considering all mullet and/or seatrout fishermen, the same basic pattern of effects emerged as when the individual policies were imposed. In terms of mullet, elimination of the roe season reduced total statewide landings of all species by nearly 50 percent (Table 8), while revenues were reduced by approximately 41 percent. The impact from imposing monthly roe season limits or a 50 percent reduction in roe season landings were essentially the same in terms of landings and revenue. Option 2 would reduce landings and revenue by approximately 21 and 18 percent, while Option 3 would reduce landings and revenue by 22 and 19 percent. The reductions in seatrout landings of from 10 to 50 percent would reduce total landings of all species from 0.6 to about three percent. Revenues would be reduced by the same scenario, from approximately two to nine percent.

Table 8 .--First round effect of combinations of selected landings restrictions on total fishermen landings and revenue, all Florida counties.

Changes in Landings

Restrictions seatrout	Restrictions on roe season[a]							
	No changes		Roe season eliminated		Quota based on three month pre-roe season average		50 % quota imposed on roe season	
Percentage of 1987 seatrout landings	Landings	Change	Landings	Change	Landings	Change	Landings	Change
	(lbs.)	(%)	(lbs.)	(%)	(lbs.)	(%)	(lbs.)	(%)
100	32,957,672	0.00	19,872,807	-49.54	26,648,101	-21.17	26,615,240	-22.04
90	32,767,957	-0.58	19,683,092	-50.43	26,458,386	-21.88	26,225,525	-22.75
80	32,578,242	-1.16	19,493,377	-51.34	26,268,671	-22.59	26,035,810	-23.67
70	32,388,527	-1.74	19,303,663	-52.25	26,078,956	-23.30	25,846,095	-24.19
60	32,198,812	-2.33	19,113,948	-53.17	25,889,241	-24.02	25,656,380	-24.91
50	32,009,097	-2.92	18,924,233	-54.10	25,699,526	-24.75	25,466,665	-25.64

Changes in Revenue

Restrictions on seatrout	Restrictions on roe season[a]							
	No changes		Roe season eliminated		Quota based on three month pre-roe season average		50 % quota imposed on roe season	
Percentage of 1987 seatrout landings	Revenue	Change	Revenue	Change	Revenue	Change	Revenue	Change
	($)	(%)	($)	(%)	($)	(%)	($)	(%)
100	10,620,490	0.00	7,009,067	-40.97	8,879,048	-17.86	8,814,778	-18.58
90	10,432,672	-1.78	6,821,249	-43.56	8,522,194	-21.92	8,457,925	-22.67
80	10,244,854	-3.60	6,633,431	-46.22	8,202,904	-25.69	8,138,634	-26.46
70	10,057,036	-5.45	6,445,614	-48.93	7,921,177	-29.12	7,856,908	-29.91
60	9,869,219	-7.33	6,257,796	-51.70	7,677,014	-32.17	7,612,745	-32.99
50	9,681,401	-9.25	6,069,978	-54.53	7,470,415	-34.82	7,406,145	-35.66

[a] No. of fishermen affected: 4,889

Source: DNR trip ticket data, 1987.

The landings and revenue impacts from the combination of the two sets of policies were somewhat similar; however, the roe season restrictions obviously dominated the effects. For example, imposing the quotas on monthly roe season landings, while simultaneously reducing seatrout catch from 10 to 50 percent, resulted in reductions of total statewide revenue ranging from 22 to 35 percent, respectively. The results were approximately the same for the 50 percent quota on the roe season catch. Eliminating the roe season and reducing the seatrout catch in 10 percent increments produced decreases in total statewide revenues of from 44 to 55 percent.

Considerable variation was exhibited among Florida counties in terms of the "first-round" effects which result from the various policy combinations. This variation is a manifestation of the differences among counties in terms of seasonal production of mullet and the overall importance of mullet and seatrout to the finfish industry in each county.

CONCLUSIONS

Long-term production trends for black mullet and spotted seatrout show similar patterns. Examination of the period 1977-1988 shows an upward trend in landings of both species until the early 1980s, followed by a general decline through 1988. From 1981 to 1988, black mullet landings declined by 38 percent and spotted seatrout by nearly 52 percent. These recent declines may be part of a long-term biological cycle, which may reverse on its own, but they may also be the result of reduced fishing effort. They may also be an ominous signal that the fishery is being depleted. In any case, trends in landings of both mullet and seatrout should be monitored closely over the next few years so that appropriate regulatory actions can be taken if deemed necessary.

Considerable heterogeneity was found to exist among fishermen, in terms of species mix, volumes and values landed, full- versus part-time involvement, and regional distribution. The majority of the production of mullet and seatrout, however, is produced by a small percentage of the total number of fishermen. Thus, any policy considerations oriented toward the in-shore fishery should recognize the complexity of the production sector, particularly landings distributions among fishermen for specific species.

Market channels for mullet are also relatively complex because of the many product forms derived from mullet. Potential regulatory policies could drastically affect some channels, e.g. those handling roe and split carcasses, leaving others practically unaffected. The market channels delineated and product flows described by this study can be useful in assessing the implications of alternative policies in terms of numbers of firms affected and the relative impacts at both the primary and secondary handler levels.

The market structure found at the primary handler level, from the standpoint of buyer concentration, indicates a competitive environment on a regional basis. However, buyer concentration faced by fishermen may be great in some locales, usually a result of a limited fishery and geographic isolation rather than horizontal integration. In terms of seller concentration, relatively large numbers of firms handling mullet, regionally and statewide, are an indication of competitive marketing as

well. The effects of resource management policies would probably impact larger primary handlers to a greater degree but, even so, effects would be spread over a relatively large number of firms.

A benefit of this study is in providing a detailed description of the industry to aid policy development. The analysis of fishermen profiles may be particularly useful in assessing some basic policy alternatives. The findings of the analysis on fishermen landings and revenue profiles indicate few fishermen land the majority of mullet and seatrout. For example, 50 percent of the total landings for each species is landed by six and four percent of the total number of mullet and seatrout fishermen, respectively. Such distribution may lead to disproportionate impacts among fishermen as a result of species-specific trip quotas or bag limits administered at the individual level.

An overall quota or production limit administered for a given species on a statewide basis may have disproportionate impacts at the fisherman level as well because of varying degrees of specialization. For example, those individuals more dependent on roe mullet may experience a greater impact from roe season restrictions than fishermen who are more dependent on trout or non-roe mullet landings. In addition, a statewide quota may have disproportionate impacts on a regional basis, as some counties also vary in their dependence on seatrout or mullet production. Also, failure to recognize seasonal differences in landings among counties, e.g. roe mullet abundance, could adversely affect southernmost counties if a statewide quota is imposed. Inequities could result if the statewide quota were to be first achieved by production in northern counties, where the roe season begins earlier.

In future research, access to detailed Trip Ticket data could greatly improve the detail and accuracy of policy analyses attempted by this study. While the Florida Department of Natural Resources was most cooperative in providing aggregate Trip Ticket data, confidentiality policies precluded analyses of Trip Tickets on an individual fisherman basis. Refined policy analyses based on individual Trip Tickets should be made prior to implementation of specific resource management policies.

Other areas that should be examined are demand analysis at the dockside level, and production costs and supply response at the fisherman level. Additionally, market research and development directed toward improving the utilization of mullet, particularly split carcasses, could substantially improve fishermen's incomes and consumers' welfare as well. Finally, future policy development would benefit from an integration of economic analyses, such as presented in this study, with ongoing biological studies currently being conducted by various state resource management agencies.

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Species Identification of Retail Snapper Fillets in Florida

Yun-Hwa P. Hsieh, Paul F. Kirsch, and Betsy B. Woodward
Florida Department of Agriculture and Consumer Services
Food Laboratory, Division of Chemistry
3125 Conner Boulevard, Tallahassee, FL 32399-1650

Electrophoretic techniques have been widely used to study sarcoplasmic proteins. The technique involves the migration of charged protein species in a controlled electrical field so that each protein species migrates to the pH at which the molecular charge is neutral, its isoelectric point. At that point the protein no longer migrates and "focuses" or concentrates into discrete bands. For fish sarcoplasmic proteins, the electrophoretic patterns are characteristic of the fish species similar to the way that finger prints are characteristic of individuals. Electrophoresis has been used in the Food Laboratory of the Department of Agriculture and Consumer Services for twenty years to identify fish species.

Initially, a cellulose acetate strip electrophoresis technique was used which resulted in a pattern with fewer characteristic bands and identification frequently was not as well defined. The newer isoelectric focusing techniques were developed and applied to fish by Lundrstrom at the National Marine Fisheries Laboratory in Glouster, Massachutes in the late 1970's (1). The technique was collaboratively studied and recommended as an Official Method by the Association of Official Analytical Chemists in 1980 (2). Subsequently, the Food Laboratory sent an analyst to the NMFS laboratory in Massachutes and incorporated the method for use in fish identification in 1983.

Under Chapter 500.02(1), Florida Statutes, the department is charged with "protecting the purchasing public...from injury by merchandising deceit...". This means that food offered for sale must be properly represented in its labeling. Additionally, the U. S. Food and Drug Administration Compliance Policy Guide 7108.21 (3) provides that "the labeling or sale of any fish other than Lutjanus campechanus as "red snapper" constitutes a misbranding in violation of the Federal Food, Drug and Cosmetic Act".

The study presented here represents official samples examined for compliance with the above regulations during fiscal year 1988/89 using isoelectric focusing to identify fish fillets as to species and to confirm the label declaration of species as correct.

MATERIALS AND METHODS

Fish samples:
Authentic fishes including red snapper (Lutjanus campechanus), lane snapper (Lutjanus synagris), gray snapper, also known as mangrove snapper, (Lutjanus griseus), mutton snapper (Lutjanus analis), mahogany snapper (Lutjanus mahogoani), vermillion snapper (Rhomboplites aurorubens) yellowtail snapper (Ocyurus chrysurus), and hogfish also know as "hog snapper" (Lachnolaimus maximus) were authenticated by the Florida Department of Natural Resources and are maintained frozen in the Food Laboratory. Scarlet snapper and malabar snapper were collected and donated by Winn Dixie, Jacksonville, Florida. Whole

red rock fish was purchased from a commercial dealer. These three are pending authentication. All the snapper samples were official samples, collected and sent to the laboratory by state food inspectors. They represent snappers offered for sale at retail supermarkets and fish dealers across Florida during FY 1988/89 year. The fish were cut into small (about 5g.) pieces, put into whirl-pak bags (Fisher Scientific) and stored at -20 C. until used.

Sample preparation:
Frozen fish tissue was partially thawed. A small portion of about 0.5 g. was diced and inserted into a 1.5 ml microcentrifuge tube (Fisher Scientific), and centrifuged in a micro-centrifuge (Model 235B, Fisher Scientific) at a fixed speed of 13,750 rpm for 3 minutes. The supernantant containing sarcoplasmic proteins was used for the thin layer isoelectric focusing analysis.

Thin layer isoelectric focusing (IEF):
IEF was performed according to the modified procedures of Lundstrom (2) using a Bio-Rad electrophoresis cell (Model 1415) connected to a constant temperature circulator (Precision RDL 20) at 5 C. and a Bio-Rad power supply (Model 1420B). Five uls of each protein sample or standard extract were applied to a small adsorbent sample application piece on a LKB Ampholine PAG plate gel (pH 3.5-9.5). The protein standards (Broad range pI 4.6-9.6, Bio-Rad, Richmond, CA) contained: cytochrome C, pI 9.60; lentil lectin-basic band, pI 8.60, -middle band pI 8.40, -acid band pI 8.20; human hemoglobin C, pI 7.50, human hemoglobin A, pI 7.10; equine myoglobin, pI 7.00; human carbonic anhydrase, pI 6.50; bovine carbonic anhydrase, pI 6.00; beta-lactoglobulin B, pI 5.10; and phycocyanin, pI 4.65. Proteins were first separated for 30 min at 10 watts. Then sample application pieces were removed and separation continued for 20 min at 20 watts. After removal of the power, the gel was fixed, stained with hot Coomassie blue R-250 (Bio-Rad Laboratories at 60 C.; destained; preserved and dried as described by Lundstrom (2).

RESULTS AND DISCUSSION

The seven distinctive protein patterns of the authentic snappers as well as that of authentic hogfish are shown in Figure 1. Hogfish is frequently referred to as "hog snapper", but it is not in the snapper family. Hogfish was included in this study because it has been sold as "snapper" and even as a substitute for red snapper due to the regional use of the name, "hog snapper", and its red-orange color. Two imported red skinned snappers: scarlet snapper and malabar snapper; and one pacific red rock fish which have not been officially authenticated were also included for comparison as standards. Four samples (column 12-15, Fig. 1) were individually compared with all the standard species' protein patterns (column 2-11, Fig 1). All of these four samples were labeled as red snapper, but none of them match the authentic red snapper (column 2, Fig 1). Samples # 1-3 match the protein pattern of scarlet snapper (column 8, Fig 1) and sample #4 closely resembles lane snapper (column 5, Fig. 1). The overall results for the retail snapper fillets collected throughout Florida in FY 1988/89 are summarized in Table 1. Of the 70 official fish samples collected, 50 were labeled as red snapper; 3 as mutton snapper; 1 as yellowtail snapper; 2 as vermillion snapper; 7 as "snapper"; 3 as scarlet snapper; 1 as scarlet red snapper; 1 as "pacific red snapper"; 1 as "imported red snapper"; and 1 as malabar snapper. Only 18 of the 50 red snapper samples (36%) were in fact confirmed as true red snapper (Lutjanus campechanus). These results indicate that 64% of the red snapper samples were mislabeled, while mutton snapper, yellowtail snapper and vermillion snapper were all correctly labeled.

The type of fish which did not match any protein patterns of the available authentic species was designated as an unknown group. The samples labeled as "scarlet snapper", "scarlet red snapper", and "pacific red snapper" appeared to have identical protein patterns and were grouped as unknown #1. One sample labeled as "imported red snapper" had a different protein pattern from the others and so was designated as unknown #2. Samples which match the "malabar snapper" protein pattern were designated as unknown #3. The last unidentified group was unknown #4.

Because red snapper is so likely to be substituted, some fish dealers label the fish simply as "snapper". The seven samples labeled as "snapper" in our survey were all red-skinned fillets. Among them one was identified as lane snapper; the remaining four were unidentified. None of them was genuine red snapper.

As shown in Table 2, among the thirty-two mislabeled red snappers, three were identified as mutton snapper; one as yellowtail snapper; one as vermillion snapper; three as lane snapper; six as gray snapper; nine matched unknown #1; four matched unknown #2; three were grouped as unknown #3 and two as unknown #4. The species accounting for the largest number of red snapper substitutions had the same protein pattern as the so-called "scarlet snapper"; "scarlet red snapper" or "pacific red snapper." The second largest number of substitutes was gray snapper. Nomenclature on the fish market is clearly a problem. The same species of fish is frequently identified by several different name, especially for those imported red-skinned fish. Scarlet snapper is imported from Thailand and malabar snapper is imported from Taiwan. Both are imported pacific red-skinned snappers. There is no such fish as "imported red snapper", "scarlet red snapper" or "pacific red snapper". The word, "red", is attached for marketing purpose. Hopefully utilization of "The Fish List" (4), the U. S. Food and Drug Administration's guide to acceptable market names will serve to standardize fish nomenclature.

Figure 2 shows a juxtaposed comparison of several samples with the authentic and non-authenticated standards. All of these samples were labeled as "red snapper", but none match the authentic red snapper protein pattern. Samples # 16, #23, and #30 match the pattern of scarlet snapper (column 14,); #78 matches vermillion snapper (column 4); #73 matches mutton snapper (column 6); #04 matches lane snapper (column 8); #25 matches gray snapper (column 10) and #22 matches malabar snapper (column 16).

Market prices of the fish samples collected were not available, therefore no attempt was made to relate economics with the accuracy of the labeling, although there are obviously economic considerations in fish substitutions. Red snapper is considered highly susceptible to substitution for several reasons: it is expensive, popular and in short supply. Many of the substitutes are inexpensive, some have comparable taste, and many have red skins that resemble the red snapper. Virtually it is impossible to tell most of them apart once cut into fillets; and species cannot be identified for sure without laboratory analysis. Therefore fish retailers may be unaware of the mislabeling problem and are at the mercy of their fish distributors to supply the real thing, especially for pre-cut fish fillets. Unlike meat and poultry, seafood is not subject to mandatory government

inspection. In 1985, the National Fisheries Institute asked Congress to mandate the design of a seafood inspection system based on modern Hazard Analysis Critical Control Point (HACCP) concept recommended by the National Academy of Sciences. The design of this seafood surveillance program has been underway for the past two years. This should be the time that an improved regulatory inspection system be put in place to ensure the quality, wholesomeness and accurate labeling of seafood sold to consumers.

Special acknowledgement and thanks is given to Charles Futch, Florida Department of Natural Resources, for his assistance in authenticating whole fish as to species.

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Table 1. Results of the species identification of retail snappers sold in Florida in FY 1988/89 by thin-layer isoelectric focusing methods.

Label Designation	No. of Samples Obtained	No. of Samples Identified	Percent Misbranded
Red Snapper	50	18	64
Mutton Snapper	3	3	0
Yellowtail Snapper	1	1	0
Vermillion Snapper	2	2	0
Lane Snapper	0	0	
Gray Snapper	0	0	
"Snapper"	7	3 a	
Unknown #1		5 a	
Scarlet Snapper	3		
Scarlet Red Snapper	1		
Pacific Red Snapper	1		
Unknown #2		a	
Imported Red Snapper	1		
Unknown #3		a	
Malabar Snapper	1		
Unknown #4		2 a	
TOTAL	70	33	

a Additional samples in these groups compare with the unauthenticated standard species and are pending confirmation.

TABLE 2. Distribution of Actual Species in the 50 "Red Snapper" Labeled Fillets.

Species Name	No. Identified	Percent
Red Snapper	18	36
Mutton Snapper	3	6
Yellowtail Snapper	1	2
Vermillion Snapper	1	2
Lane Snapper	3	6
Gray Snapper	6	12
Scarlet Snapper (Unknown #1)	9	18
Malabar Snapper (Unknown #2)	4	6
Unknown #3	3	8
Unknown #4	2	4
TOTAL	50	100

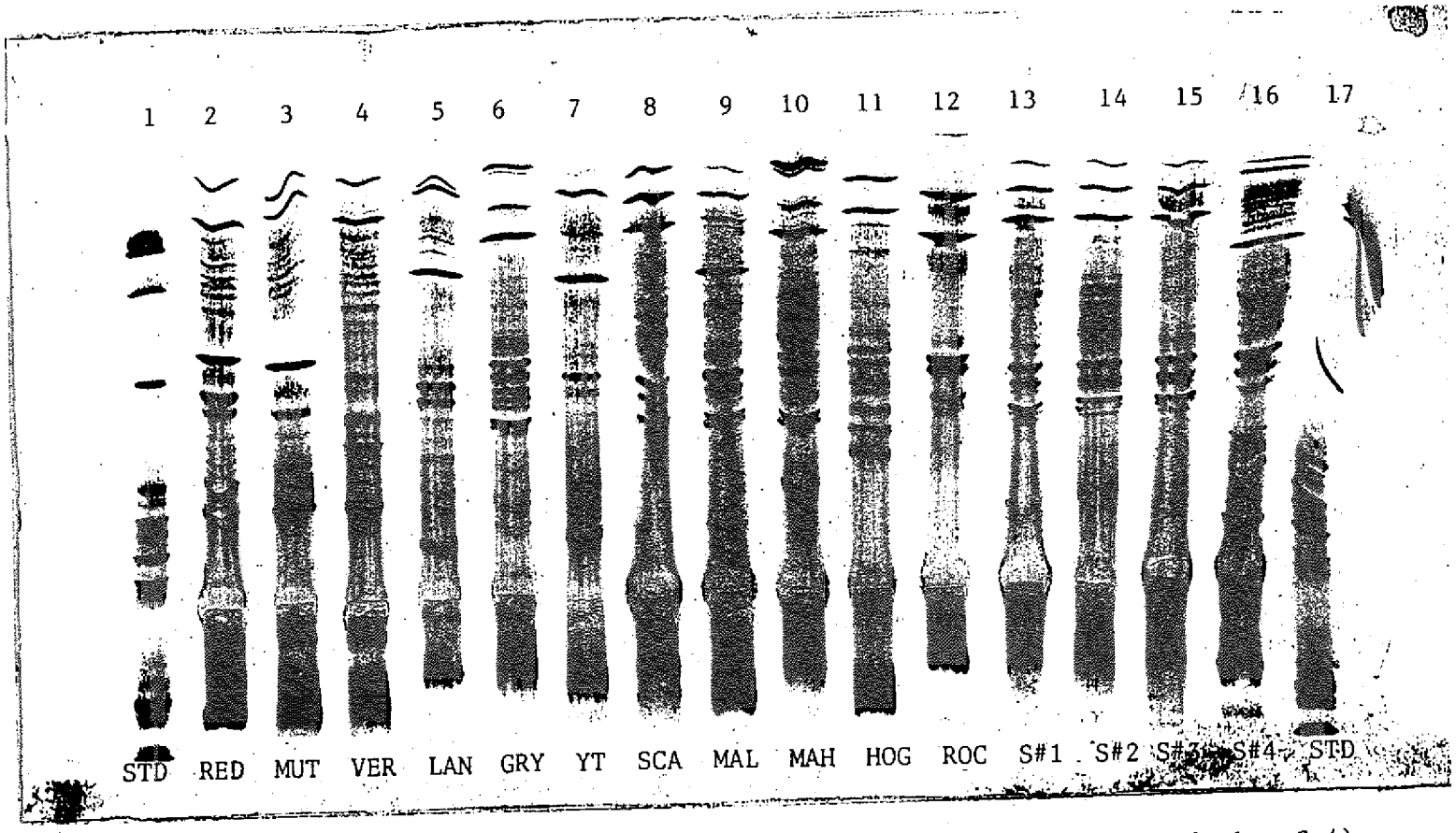

Figure 1. Thin layer IEF PAG Gel, Authentic snapper species with Samples S-1 thru S-4)

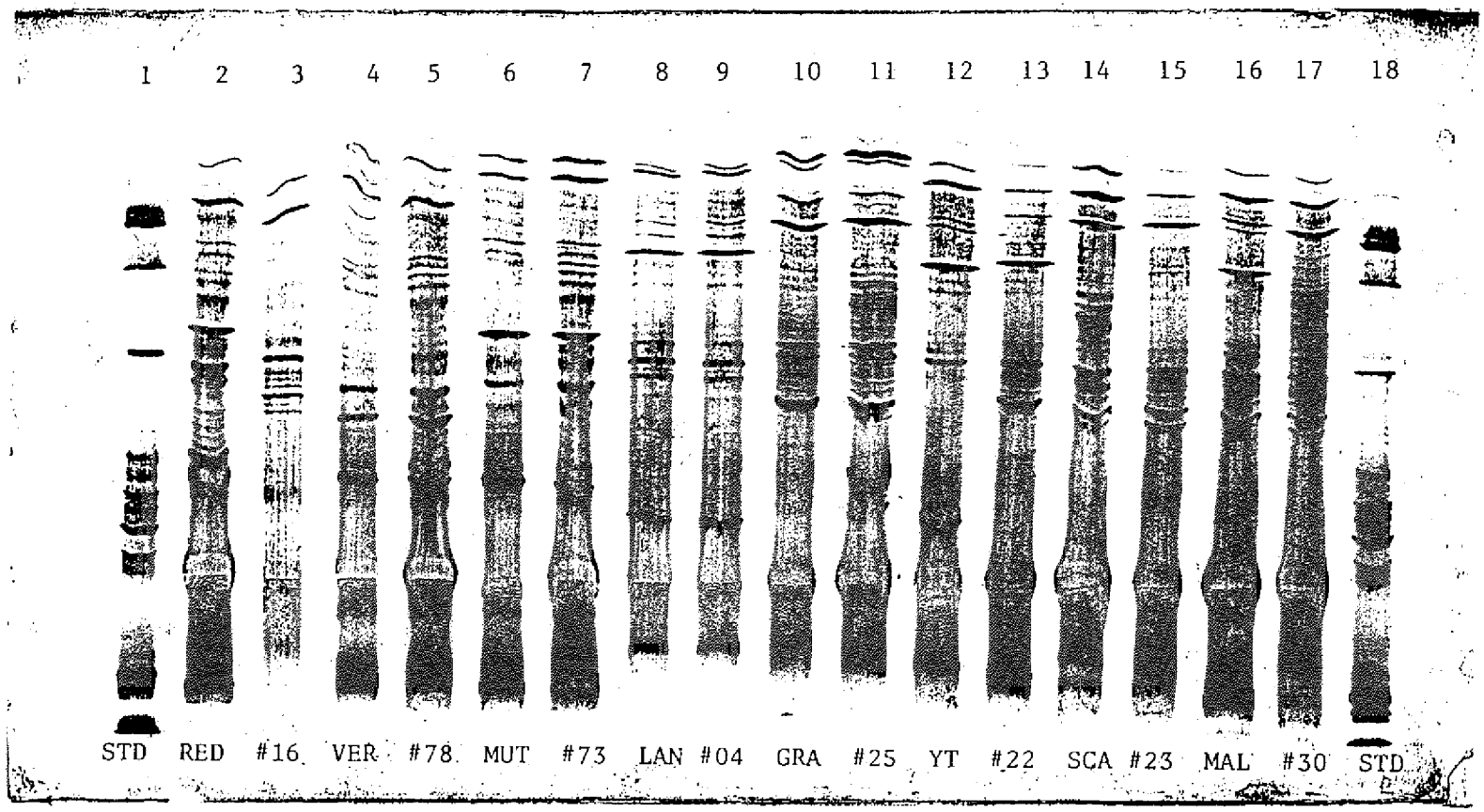

Figure 2. Thin layer IEF PAG Gel, Authentic species and samples # 16, 78, 73, 04, 25, 22, 23 and 30.

ABSTRACTS

EXTRACTION AND CHARACTERISTICS OF FLAVOR CONCENTRATE FROM MECHANICALLY RECOVERED CRAB MEAT

M. Nieto, A. Beltran, and R.T. Toledo

Food Science Department

University of Georgia

Athens, GA 30602

Crab flavor concentrates were prepared by washing commercially produced mechanically recovered (Baader) crab meat with salt water followed by enzyme digestion using chymotrypsin. An increase in salt soluble components occurred with increasing time of digestion, and this fraction appears to level off at around 35 percent by weight of the initial mass within 2 hours of digestion. Dilution of the crab meat with water to 30 percent total solids was necessary to reduce consistency and enhance enzyme activity. Increasing enzyme concentration increased rate of solubilization but not the total conversion. The residue after filtration of the digested material showed small fragments of shell, indicating that this procedure may also be used analytically to determine the effectiveness of the deboning process in preventing shell inclusion in the mechanically recovered meat. The extract after digestion had excellent crab flavor with no bitter aftertaste.

MONOCLONAL ANTIBODY-BASED ENZYME IMMUNOASSAY OF ROCK SHRIMP

C.-I. Wei, H. An, M.R. Marshall, W.S. Otwell, K.-J. Kao, and P.A. Klein

Food Science and Human Nutrition Department

Department of Pathology and Laboratory Department

University of Florida

Gainesville, FL 32611

Rock shrimp-specific monoclonal antibody (4H2-10D3) was applied in ELISA tests to detect and quantitate rock shrimp in various seafood and meat samples. Under the optimized testing conditions for ELISA, the use of this McAb in a blind study correctly differentiated rock shrimp from 23 other seafood and meat samples. Furthermore, the presence of rock shrimp as low as 4.3 ng on the average could be detected in sample mixtures containing various seafood or meat samples. The results indicated this McAb can be used to quantitate rock shrimp in mixture samples.

A BIOCHEMICAL METHOD TO DISTINGUISH WILD FROM CULTURED FISH

M. Jahncke, T.T.J. Smith, and G. Seaborn

NMFS Charleston Laboratory

P.O. Box 12607

Charleston, SC 29412

or

SCWMRD

P.O. Box 12559

Charleston, SC 29412

Research is currently underway at the Charleston Laboratory to develop a biochemical method to distinguish wild (poached) from cultured (farmed) fish. The objective is to use fatty acid composition differences to differentiate wild from cultured fish.

Our current research indicates that in addition to linoleic acid (18 : 2n6), differences in the concentrations of other long chain polyunsaturated fatty acids such as; linolenic acid (18 : 3n3), arachidonic acid (20 : 4n6), docosapentaenoic acid (22 : 5n6) and docosahexaenoic acid (22 : 6n3) can also be used to help distinguish wild from cultured fish.

PRELIMINARY COMPARISON OF THE EFFECT OF LACTIC ACID VERSUS LACTIC/ACETIC ACID MIXTURE ON THE INHIBITION OF GROWTH AND RESUSCITATION OF LISTERIA MONOCYTOGENES

M. Barbier, W.S. Otwell, and G. Rodrick

Food Science and Human Nutrition Department

University of Florida

Gainesville, FL 32611

In recent years, the interest in control of Listeria monocytogenes has grown because of the frequency of incidence in a wide variety of foods. Lactic acid has shown to offer some control of the organism. Recently, investigators have questioned whether a combination of antimicrobials could be more effective than a single agent. A synergistic interaction has been demonstrated between lactic and acetic acid on the decontamination of beef carcasses. The probable interaction was due to the potentiation of acetic acid by lowering the pH by lactic acid and thus increasing the undissociated fraction. Likewise, vacuum package pork treated with 1 percent acetic/1 percent lactic acid was more effective against aerobic, anaerobic, facultative aerobic bacterium and lactobacilli than just 1 percent acetic acid.

These and other recent studies gave rise to the idea that perhaps a mixture of lactic and acetic acid could be more effective against Listeria monocyogenes than lactic acid alone. An in vitro study of the effect of treatment of cultures of Listeria with lactic, acetic and the mixture examines the expression of the microorganism after frozen storage for three days, 1, 2, 3, and 4 weeks, followed by a period of resuscitation to simulate treatment of foods following processing. The final results are incomplete at this time.

QUALITY AND STABILITY OF FRANKFURTERS CONTAINING MINCED FISH WITH RED MEATS

M. Hale, J. Gooch, and M. Jahncke

National Marine Fisheries Service

Charleston, SC 29412

Frankfurter samples containing mince, washed mince or surimi from several fish species were prepared at the Charleston Laboratory as part of a cooperative study by NMFS and USDA. An improved analytical procedure developed by the USDA shows that nitrosamine formation in fish and red meat franks cured with nitrite is generally low. Sensory and instrumental evaluations of frankfurters containing pollock or menhaden are reported as a function of minced fish form, concentration, and storage condition.

MICROBIOLOGICAL HAZARDS IN SEAFOODS

L.R. Beuchat and R.E. Brackett

Department of Food Science and Technology

Georgia Agricultural Experiment Station

University of Georgia

Griffin, GA 30223

Seafoods are among the foods most often implicated in microbial foodborne disease. This, together with the growing market for these foods, has stimulated an increased concern for seafood safety. The general microflora of seafoods normally consists of non-pathogenic, psychrotrophic spoilage bacteria. However, common and less common pathogenic microorganisms also occasionally contaminate seafoods.