PURIFICATION AND CHARACTERIZATION OF A TRYPsin-LIKE ENZYME FROM
THE HEPATOPANCREAS OF CRAYFISH (PROCAMBARUS CLARKII)

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INTRODUCTION

Proteolytic enzymes have been used in a number of industrial applications including
laundry detergents, feed, cheese making, meat tenderizing, fermented sauces, and the
production of pharmaceuticals (18). Proteolytic enzymes obtained from livestock offal do
not adequately meet the demand on a world basis and the future availability of traditional
enzyme sources is sometimes dependent on the political and agricultural policies (10).
Moreover, traditional animal enzyme sources have been restricted to relatively few species,
namely, bovine and porcine offal.

Recent interest has developed concerning the proteases found in stomachless
marine organisms. Stomachless marine organisms are deprived of the acid denaturation
which takes place in the stomach, making the protein in the feed more amenable to
subsequent degradation in the intestine by trypsin, chymotrypsin and other proteases or
peptidases. Indeed, trypsin and chymotrypsin from higher vertebrates do not hydrolyze
native proteins or do so at a very low rate compared to denatured proteins (15, 19).
Additional evidence showed that a protease from crayfish hydrolyzed native ribonuclease
and inactivated native lactate dehydrogenase while proteases from species having a
stomach were not effective (22).

This paper describes the purification and characterization of a trypsin-like enzyme
from the hepatopancreas of Louisiana swamp crayfish. The properties of this crayfish
enzyme are also compared to those of bovine trypsin and trypsins from other marine
organisms.

MATERIALS & METHODS

Preparation of crayfish extract

Louisiana swamp crayfish (Procambarus clarkii) purchased live from a local seafood
store were decapitated, and the hepatopancreas removed, rapidly frozen in liquid nitrogen
and ground to a powder using a Waring blender. The powdered hepatopancreas was
stored at
-70°C.

Twenty-five grams of the powdered hepatopancreas was homogenized for 1 min
in 125 mL of ice-cold distilled water and 30 mL of tetrachloromethane using a Waring
blender. The homogenate was centrifuged at 27,000 x g for 20 min at 4°C and the
supernatant was collected for enzyme purification.
Purification of protease

Unless otherwise specified all steps were carried out at 4°C. The defatted crude extract was subjected to ammonium sulfate fractionation and the precipitate from the 30-70% saturation range was collected and resuspended in 25 mL of 10 mM Tris-HCl buffer (pH 7.0) containing 0.7 M (NH₄)₂SO₄. This sample was applied slowly (0.2 mL/min) to a Phenyl Sepharose CL-6B column (1.6 x 40 cm, Pharmacia). Elution was accomplished with a 0.7-0 M linear gradient of (NH₄)₂SO₄ in 10 mM Tris-HCl (pH 7.0). Material passing through the column was collected in fractions of 4 mL/tube using a Bio-Rad fraction collector (model 2110). Absorbance at 280 nm was recorded using a spectrophotometer. Fractions absorbing light at 280 nm were assayed for esterase activity according to the method of Hummel (14).

Fractions eluting between 120 and 160 mL were pooled and concentrated to 3 mL via ultrafiltration utilizing a 50K omegacell filter (Filtron) and applied to a 2.5 x 50 cm Sephadex G-100 column (Pharmacia) which was equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.0) at 0.2 mL/min. Material passing through the column was collected in 4 mL/tube fractions and checked for absorbance at 280 nm and esterase activity. Fractions eluting between 128 and 160 mL were pooled and applied to a 1.6 x 40 cm column packed with DEAE cellulose which had been equilibrated with 50 mM Tris-HCl (pH 7.0). Elution was accomplished using a 0-1 M linear gradient of NaCl in 50 mM Tris buffer at 0.2 mL/min. Material passing through the column was collected in 4 mL/tube fractions, and checked for absorbance at 280 nm and esterase activity. Fractions eluting between 120 and 168 mL were pooled and stored at -20°C for further studies.

Activity measurement

Esterase activity of the protease was assayed using tosylarginine methyl ester (TAME) as substrate. Tosyl arginine released at 25°C from TAME was measured by an increase in absorbance at 247 nm in a spectrophotometer under the assay conditions in which 100 μL enzyme preparation was mixed with 0.3 mL 0.01 M TAME, and 2.6 mM Tris-HCl (pH 8.1) containing 11.5 mM CaCl₂ (14). One TAME unit of activity was defined as ΔA₂₄₇ₙₘ/min x 1000 x 3 divided by 540; where 540 is the extinction coefficient of tosyl arginine (1).

Amidase activity was determined according to the method of Erlanger et al. (5) using Nα-benzoylarginine-p-nitroanilide (BAPA) as substrate. A 200 μL aliquot of the isolate was added to 2.8 mL of 1 mM BAPA in 0.05 M Tris-HCl (pH 8.2) containing 0.02 M CaCl₂ and the release of p-nitroanilide was measured at 410 nm at 25°C. One BAPA unit of activity was defined as ΔA₄₁₀ₙₘ/min x 1000 x 3 divided by 8800; where 8800 is the extinction coefficient of p-nitroanilide (5).

For assays with p-toluenesulfonyl-L-lysine methyl ester (TLME) and benzoyl-tyrosine ethyl ester (BTEE), conditions were the same as for TAME except for the substitution of the indicator substrate at a final concentration of 1 mM. A unit of TLME and BTEE activity is defined as the increase in absorbancy of 0.001/min at 25°C (14).

The influence of pH on protease activity and stability

Protease activity as influenced by pH was determined using TAME as substrate. The pH optimum for TAME hydrolysis was determined by preparing the substrate in the various buffer solutions: 0.1 M citrate-HCl, pH 2.0; 0.1 M citrate-NaOH, pH 3.0; 0.1 M citrate-NaOH, pH 4.0; 0.1 M citrate-NaOH, pH 5.0; 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 9.0; and 0.1 M
glycine-NaOH, pH 10.0, and allowing hydrolysis by the enzyme to proceed as above. The kinetic constants ($K_{m}$ and $V_{max}$) were then estimated for each pH by Lineweaver-Burk plots at various concentrations of TAME (0.3 - 1.0 mM). Data were weighted using "EZ-FIT" software as described by Perrella (21).

Stability of protease at various pH's was determined by preincubating 25 μL of the enzyme in 275 μL of the various buffer solutions in an ice bath for 5 min prior to assaying for esterase activity.

**Temperature stability**

Thermostability of the protease was determined by equilibrating the enzyme at various temperatures for 30 min, cooling rapidly in an ice bath for 5 min and then adding substrate at 25 °C.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis**

Electrophoresis was performed under reducing conditions (17) utilizing a 5% stacking gel and a 12% separating gel. Proteins were detected by staining with 0.1% Coomassie brilliant blue R-250 dissolved in a solution containing 40% methanol and 10% acetic acid in water. Molecular weights were estimated according to the method of Weber and Osborn (27) using a Sigma low MW standard kit containing bovine serum albumin (66 KD), egg albumin (45 KD), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 36 KD), carbonic anhydrase (bovine erythrocytes, 29 KD), trypsin (24 KD), soybean trypsin inhibitor (20.1 KD), and α-lactalbumin (14.2 KD).

**Isoelectric Focusing**

Precast agarose isoelectric focusing gels (IsoGel), pH 3-10, 0.6 mm thick, were purchased from FMC Bioproducts. Purified protease (5μL) and isoelectric focusing standards (Broad pl kit, pH 3-7, Pharmacia) were loaded onto strips of blotting paper (Schleicher and Schuell Inc.) placed in the center of the gel and run for 2 h at 8 W (500 V limiting) on a Hoefer Isobox flatbed system using 10 mM phosphoric acid as the anode solution and 20 mM sodium hydroxide as the cathode solution. Gels were stained in 0.1% solution of Coomassie brilliant blue R-250 in 25% ethanol and 9% glacial acetic acid.

**Protein Determination**

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

**Inhibition Study**

Enzyme inhibitors including phenylmethyl sulfonil fluoride (PMSF), tosyl lysine chloromethyl ketone (TLCK), tosyl phenylalanine chloromethyl ketone (TPCK), aprotinin, soybean trypsin inhibitor (SBTI), benzamidine, 1,10-phenanthroline, and 8-hydroxyquinoline on the activity of crayfish protease (CP) were separately determined by incubating each inhibitor with the enzyme at 25°C for 30 min before application of TAME and measurement of the initial rate of enzyme activity.
Activation study

The influence of the bivalent ions Ca\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Hg\(^{2+}\) and Ag\(^{+}\) on the CP activity was separately determined by incubating each ion with the enzyme at 25°C for 30 min before application of TAME and measurement of enzyme activity.

Antibody preparation

Immunological cross reactivity between proteases was tested by using purified protease from crayfish as an antigen to induce antibody production in hens. Animals were injected with 100 μg of the enzyme, and boosted every 2 weeks with an additional 100 μg. Antibodies were purified from egg yolk by the method of Polson et al. (23). Yolk was separated from the white, diluted with 4 volumes of 0.1 M sodium phosphate buffer (pH 7.6), and mixed with 3.5% (w/v) polyethylene glycol (PEG) until a homogeneous mixture was obtained. The suspension was centrifuged at 5,000 x g for 20 min. To the supernatant was added 8.5% (w/v) PEG, and mixed. After standing for 10 min, the mixture was centrifuged at 5,000 x g for 25 min. The precipitate obtained was dissolved in the phosphate buffer at a volume equal to 2.5 times that of the egg yolk. The mixture was made to 12% PEG (w/v) and let to stand for 10 min before centrifugation at 5,000 x g for 25 min. The pellet was dissolved in phosphate buffer at a volume equal to 0.25 times that of the egg yolk, cooled to 0°C and then a same volume of 50% ethanol (v/v) precooled at -20°C was added. After centrifugation at 10,000 x g for 25 min, the pellet obtained was dissolved in phosphate buffer at a volume equal to 0.25 times that of the egg yolk. The antibody solution was dialyzed with stirring at 4°C against 50 volumes of phosphate buffer for 24 hr, made to 0.1% sodium azide, and stored at 4°C.

ELISA procedures

Diluted antigen (1 μg/mL in 0.1 M sodium carbonate buffer, pH 9.6) at 100 μL was added to each well of an immulon plate (Fisher Scientific Co.). After incubation for 2 h at room temperature, the plate was washed four times with PBS-Tween buffer. Following the addition of diluted antibody solution (1/1000, 1/10,000 and 1/100,000 with PBS-Tween) at 100 μL per well and incubation for 2 h at room temperature, the plate was washed four times with PBS-Tween. Each well was then added with 75 μL anti-chicken alkaline phosphatase conjugate diluted 1/500 with PBS-Tween and incubated for an additional 2 h at room temperature. Following washing the plate four times with PBS-Tween, alkaline phosphatase substrate (p-nitrophenol acetate) dissolved in substrate buffer (0.1 M sodium carbonate and 0.5 mM magnesium chloride, pH 9.4) at 1 mg/mL was added (75 μL per well). Following incubation for 15 min, absorbance at 405 nm was read with an ELISA reader. The blank controls contained no antigen or antibody; the higher color intensity of the two was taken as the blank determination.

In competitive ELISA experiments, various amounts of competitors were mixed with the primary antibody solution prior to allowing them to interact with the bound antigen. Anti-CP antibody was used at 1/1000 dilution and 100 ng of CP was plated as antigen. CP, trypsin, chymotrypsin and pepsin at 0-100 ng were added as competitors to the anti-CP antibody. To exclude the possibility of antibody being digested by the proteases, either PMSF (5 mM) or benzamidine (10 mM) was added prior to incubation with antibody.
RESULTS & DISCUSSION

Purification of crayfish protease

A summary of CP purification is presented in Table 1. The specific activity of the purified CP (corresponding to 7% yield) was about 40-fold greater than that of crude extract. The yield of CP was low even though the extraction conditions (eg. pH and temperature) were in the stability range for the enzyme. The presence of other serine proteases that have high esterase activity toward TAME in the extract may have contributed to the low yield. Another possibility for the low yield could be attributed to the low content of the protease.

Table 1. Purification of crayfish protease from Procambarus clarki hepatopancreas.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units*)</th>
<th>Specific activity (units*/mg)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defatted crude extract</td>
<td>258.50</td>
<td>900</td>
<td>3.5</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>30-70% (NH₄)₂SO₄</td>
<td>75.20</td>
<td>825</td>
<td>10.9</td>
<td>92</td>
<td>3.1</td>
</tr>
<tr>
<td>HIC</td>
<td>8.90</td>
<td>696</td>
<td>78.2</td>
<td>77</td>
<td>22.3</td>
</tr>
<tr>
<td>G-100</td>
<td>1.41</td>
<td>144</td>
<td>102.0</td>
<td>16</td>
<td>29.1</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>0.40</td>
<td>63</td>
<td>157.0</td>
<td>7</td>
<td>44.9</td>
</tr>
</tbody>
</table>

*One unit catalyzes the hydrolysis of one μmol TAME/min at 25°C, pH 8.2.

Enzyme specificity

Purified CP only hydrolyzed TAME and TLME but not BAPA (Table 2). CP thus cleaves at sites next to arginine and lysine residues when coupled with methyl or ethyl esters, but not at sites next to arginine when coupled with p-nitroanilide (BAPA). Similar observations were reported with crustacean and bovine trypsins. Klimoto et al. (16) and Dendinger and O'Connor (3) reported a lack of activity when benzol-arginine naphthylamide (BANA) was used with trypsin-like enzymes from Euphausia superba and Callinectes sapidus, respectively. CP was also shown to have more than twice the activity with TAME than with TLME. It had no chymotryptic activity against BTEE.

Electrophoresis

The purified CP following ion exchange chromatography had only one single band with a molecular weight of about 33.6 KD on the SDS-PAGE gel (data not shown). This crayfish protease has a higher molecular weight than most of the other crustacean trypsin-like enzymes: crayfish, 24 KD (4); shrimp, 24 and 25 KD (8, 9); crabs, 16.7 and 20.5 KD (2); and spiny lobster, 24 KD (7). Trypsins with higher molecular weights were also isolated from krill (28-30 KD) (16) and Atlantic blue crab (33.5 KD) (3).
Crayfish protease also appeared as a single band on isoelectrically focussed gels (pH 3-10) (data not shown). It had a pl value of around 3.0 indicating that this CP is high in acidic amino acids. Zwilling et al. (29) found the trypsin-like enzyme of crayfish had a pl of 3.8, while Kimoto et al. (16) reported a value of 2.6 for Arctic krill trypsin-like enzyme. Trypsin-like enzymes from some crustacean species were reported to have more than twice as many acidic amino acids as the bovine form (26).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity *</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAME</td>
<td>157.1</td>
</tr>
<tr>
<td>LME</td>
<td>71.4</td>
</tr>
<tr>
<td>BAPA</td>
<td>0.0</td>
</tr>
<tr>
<td>BTEE</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Specific activity (units/mg protein) as described in Materials and Methods.

pH optima and stability

CP was found to have a narrow pH optimum with an estimated maximum at about pH 8.0 (Fig. 1A). Serine proteases, particularly the trypsin-like enzymes, from invertebrates (9, 12, 13, 15, 24) and vertebrates (5, 24) were reported to have similar pH optima values.

CP exhibited optimum stability over a pH range of 7.5-9.0 (Fig. 1B). This result is similar to that of most proteases thus far characterized for marine organisms. Many of these proteases are highly unstable under acidic but very stable at neutral to slightly alkaline conditions (12, 13, 15, 20, 28).

Temperature stability

This CP had a similar temperature stability as enzymes from other fish species (13, 24). Approximately 50% of the initial activity was retained after incubation at 55°C for 30 min, but only 20% remained after 30 min at 65°C (Fig. 2B). Proteases from fish thus far characterized tend to inactivate at 40-50°C (13, 22, 24).

The influence of various inhibitors on crayfish protease

PMSF has been described as a serine protease inhibitor; it reacts with the essential serine residue at the active center of proteinases (6, 11). The inhibition of CP by PMSF but not by 8-hydroxyquinoline and 1,10-phenanthroline (Table 3) suggests that CP is a serine protease rather than a metallo- or sulphhydryl-enzyme. Jany (15) demonstrated that trypsin from a stomachless bonefish was inhibited by PMSF while Hjelmeland and Raa (13) similarly observed inhibition of capelin trypsins by PMSF. The inhibition of CP by TLCK, SBTI, aprotinin and benzamidine, but not by TPCK also indicates that the enzyme has an action mechanism similar to that of trypsin-like enzymes (Table 3).
Fig. 1. pH optimum (A) and pH stability (B) of crayfish protease. Activity was measured using TAME as substrate.

Fig. 2. Temperature optimum (A) and thermostability (B) of crayfish protease. Activity was assayed using TAME as substrate.
Table 3. Effect of protease inhibitors on the esterase activity of crayfish protease.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td>PMSF</td>
<td>2 mM</td>
<td>50.6</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>89.4</td>
</tr>
<tr>
<td>SBTI</td>
<td>25 mM</td>
<td>98.5</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>99.1</td>
</tr>
<tr>
<td>TPCK</td>
<td>1 mg/mL</td>
<td>0.0</td>
</tr>
<tr>
<td>TLCK</td>
<td>2 mM</td>
<td>100.0</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0.25 TIU⁺</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td>0.50 TIU⁺</td>
<td>100.0</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>1.3 mg/mL</td>
<td>46.0</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>1 mM</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>0.0</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>1 mM</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>0.0</td>
</tr>
</tbody>
</table>

TIU stands for trypsin inhibitor units.

Inhibitors were incubated separately with equal volumes of enzyme for 30 min at 25°C before assay for residual esterase activity.

Effect of metal ions

The addition of heavy metals including Zn²⁺, Hg²⁺, and Cu²⁺ caused a substantial inhibition of CP activity (Table 4). However, Ca²⁺ and Mg²⁺ had nearly no effect on CP activity. A similar trait has also been noted for the tryptic activities of shrimp (8, 9) and spiny lobster (7). In contrast, the inhibition of the enzyme activity by calcium ions has been found in crayfish (29) and blue crab (3).
Kinetic constants

The kinetic parameters were determined from a double reciprocal plot of the initial reaction rates at various concentrations of TAME. The $K_m$ and $V_{max}$ for TAME were 0.133 ± 0.004 mM and 168.34 ± 0.70 TAME units/mg, respectively. The enzyme appeared not to act on BAPA, thus attempts to determine $K_m$ value for BAPA was not possible.

Table 4. Effect of some bivalent ions on the esterase activity of crayfish protease.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ion</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>5</td>
<td>99</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>5</td>
<td>94</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>1</td>
<td>41</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Ag$^{+1}$</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

Metal ions were incubated separately with equal volumes of enzyme for 30 min at 25°C before assaying for residual esterase activity.

Antibody preparation and immunological analysis

Four antibody samples with the highest titer as determined by ELISA were pooled and used to analyze cross-reactivity between CP and some of the well characterized proteases including bovine trypsin, bovine chymotrypsin, and porcine pepsin. As can be seen from Figure 3, color intensity was reduced greatly when 20 ng of either CP or bovine trypsin was added, and to a lesser extent when bovine chymotrypsin was added. The color intensity was further reduced in a nearly linear fashion over the range 20-100 ng. When pepsin was used as competitor, no reduction in color intensity was observed. CP and bovine trypsin are thus partially cross-reactive and have shared structural components.

CONCLUSION

The 33,600 mol. wt trypsin-like protease prepared from crayfish hepatopancreas has similar properties as those trypsin-like enzymes isolated from other crustacean. It shares structural components with bovine trypsin as determined by immunological study.

ACKNOWLEDGEMENTS

This research was supported by a grant from Tropicana Products, Inc., Bradenton, Florida.
Fig. 3. Analysis of antigenic properties of crayfish protease by competitive ELISA. Crayfish protease (100 ng) was plated and reacted with anti-CP antibody plus 0-100 ng/well of various competitors.

REFERENCES


MEASUREMENT OF LACTATE RESIDUALS ON TREATED SHRIMP
USING A YSI LACTATE ANALYZER

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Lactic acid is commonly used in the meat industry and is being used more and more in the seafood industry. Its primary function is to increase the shelflife of the product. Lactic acid is produced naturally and has GRAS status, generally recognized as safe. The most commonly used neutral salt form of lactic acid is sodium lactate. It has been noted that concentrations of sodium lactate equal to that of lactic acid have less negative taste consequences. Sodium lactate was used throughout this study.

Objective

Yellow Springs Instrument Incorporated (YSI) has developed a compact instrument in which to accurately measure lactate levels. The objectives of this study were to evaluate the use of the YSI lactate analyzer for the detection of lactate treated shrimp.

Treatment

Shrimp were untreated for the controls and soaked for various periods of time in various concentrations of sodium lactate for the three individual studies. In the first study, shrimp were treated in several concentrations of sodium lactate (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0) for 1 minute and 5 minutes. Lactate residuals were then measured on both raw and cooked samples with the YSI lactate analyzer. Secondly, shrimp were treated for 1 minute in, 1.5% sodium lactate. The treated samples and controls (untreated samples) were then held on ice in refrigerated storage (4°C) for 10 days. The lactate residuals were analyzed with the YSI lactate analyzer every two or three days.

Extraction

Key West pink shrimp, Penaeus duodorum, were used for this study. The shrimp in the treated or untreated samples were chopped and mixed. Ten gram subsamples, in triplicate, were homogenized in 25ml of 10% TCA. The samples were then centrifuged at 4000 rpm for 20 minutes. The supernatant was filtered and 25ul injected into the lactate analyzer.
Reaction Mechanism

The YSI lactate analyzer consists of a polarographic probe over which a three layer membrane is mounted, Figure 1. The sample is injected into the reaction chamber and mixed with the instrument buffer. It then passes through the polycarbonate membrane, which allows only substances with molecular weights less than 300,000 pass through. The sample then comes into contact with immobilized lactate oxidase. The lactate present is oxidized to pyruvate and hydrogen peroxide. The hydrogen peroxide passes through the cellular membrane and is oxidized at the surface of the anode, which is polarized at +0.7 volts versus Ag/AgCl, producing electrons, Figure 2. The steady state hydrogen peroxide concentration or current is linearly proportional to the concentration of lactate present in the sample.

Results

When the shrimp were treated for 1 & 5 minutes in increasing concentrations of sodium lactate, the residual levels of lactates on the product also increased, Fig 3. Also, the lactate residual on the samples soaked for one minute in the lactate solutions were less than the residuals on the samples treated for 5 minutes. The samples were also cooked and the cooked samples had less residual lactate than the raw samples which were treated in the same manner. In the storage study, the lactate residuals of the treated shrimp increased over the 10 day storage period as well as that of the controls, Figure 4.

Instrument Evaluation

The YSI lactate analyzer has many unique features. It is compact, water resistant and has a rechargeable 12 volt DC battery for field use and an AC adapter. It is controlled by a microprocessor, has an alpha-numeric liquid crystal display and menu driven interface. It is easy to use, has automatic calibration and sample result recall. A small sample size (25ul) is required. The sampling speed is approximately 90 seconds per sample. The detection range is 0 to 30 mmoles per liter and the resolution is 0.01 mmoles per liter. Linearity depends on the response of the membrane and as the membrane ages, the linearity decreases slightly. The linearity may be checked throughout an analysis by simply injecting a linearity check standard. The average standard deviation for all analyses was +/- 0.01.

Conclusions

The YSI lactate analyser is a very useful instrument in which to measure lactate residuals on treated shrimp. It is compact, easy to use, very reproducible and very little sample preparation is required. It is necessary to measure lactate residuals on treated shrimp in order to determine was concentrations of lactate to use and to evaluate the proper means of application of the lactate. With a 1 minute dip in a 3% sodium lactate solution, the residual lactate was measured to be 200 mg/100g shrimp and this amount was detected and disliked by half of the panelist.
Fig. 1  SENSOR PROBE AND ENZYME MEMBRANE

Fig. 2  LACTATE SENSOR

Electron flow is linearly proportional to the steady state H₂O₂ concentration and therefore proportional to the concentration of lactate.

RX 1  L - Lactate + O₂ → Pyruvate + H₂O₂

RX 2  H₂O₂ → 2H⁺ + O₂ + 2e⁻
Fig. 3

**SODIUM LACTATE DIP STUDY**
(Key West Pink Shrimp)

![Graph showing residual lactate (mg/100g shrimp) versus % sodium lactate solution.]

Average Standard Deviation for all analyses was +/- 0.01

Fig. 4

**STORAGE STUDY**
(KEY WEST PINKS)

![Graph showing residual lactate (mg/100g shrimp) versus time (days).]

0% Na LACTATE
1.5% Na LACTATE

Average Standard Deviation of all analyses was +/- 0.05
REFERENCES


DETERMINATION OF FISH QUALITY USING
A MICROFRESH BIOSENSO R

1LeeAnn Applewhite, Laura Ravelo, and W. Steven Otwell, PhD.
Department of Food Science and Human Nutrition
University of Florida, Gainesville, FL
and
1Florida Department of Natural Resources
Bureau of Seafood Marketing

Many physical and chemical methods have been used, other than sensory methods, to determine the freshness of fish. Ammonia, amines, volatile acids, catalase activity, and trimethylamine have been proposed (Gruger, 1972). Recently, nucleotide degradation in fish muscle has been found to be a reliable indicator of the freshness of raw fish (Scott et al., 1986). The K-value is derived by dividing the concentrations of hypoxanthine and inosine in the fish flesh by the concentrations of inosine monophosphate, inosine and hypoxanthine present.

The MICROFRESH biosensor, developed by Pegasus Biotechnology, is a quantitative device for measuring the K-value in fish tissues. The MICROFRESH biosensor is designed for both routine applications and research. The objective of this study was to evaluate the use of the Microfresh Biosensor form Pegasus Biotechnology for possible use in the seafood industry as a monitor for fish freshness.

Principle of Measurement

Immediately after the death of the fish, ATP in fish muscle degrades to uric acid through the following autolytic pathway:

ATP $\rightarrow$ ADP $\rightarrow$ AMP $\rightarrow$ IMP $\rightarrow$ HxR $\rightarrow$ Hx $\rightarrow$ X $\rightarrow$ U  where

ATP, ADP, AMP = adenosine triphosphate, diphosphate and monophosphate

IMP, HxR = inosine monophosphate, inosine

Hx, X, U = hypoxanthine, xanthine, uric acid

In many fish species, ATP and ADP disappear within 24 hours following the death of the fish. Concentrations of AMP are also negligible after this time period. Concentrations of IMP increase initially following death and then decreases corresponding to an increase in HxR and Hx. The-K value is derived from the following equation:

$$ K = \frac{[HxR] + [Hx]}{[IMP] + [HxR] + [Hx]} $$

Generally, fish having K-values of less than 0.2 are excellent and those with less than 0.4 are good. These values are hard to achieve commercially so values up to 0.65 are considered good quality for raw fish.
Reaction Mechanism

The MICROFRESH Biosensor consists of a polarographic probe which is polarized +0.7 volts with respect to a silver cathode and platinum anode. Hydrogen peroxide is oxidized at such a polarized potential. Uric acid is also electroactive and provides a limiting current at the same potential where hydrogen peroxide is oxidized. The oxidation of hydrogen peroxide produces electrons. The steady state concentration of electrons produced is linearly proportional to the concentration of inosine and hypoxanthine present in the sample.

\[ \text{H}_2\text{O}_2 \rightarrow 2\text{H}^+ + \text{O}_2 + 2\text{e}^- \]

Uric Acid + O₂ + 3H₂O ---- Allantoin + H₂O₂ + HCO₃⁻

Nucleoside phosphorylase (NP) and xanthine oxidase are immobilized on a membrane and attached to the sensing area of the polarographic probe. The probe is mounted in a sample measurement chamber. For the determination of [HxR] + [Hx], the sample is injected directly into the detection chamber. The sample comes in contact with the immobilized enzymes on the membrane. For the determination of [Hx] + [HxR] + [IMP], the sample is first transferred to the test tube containing immobilized nucleotidase. The IMP in the sample is converted to HxR. This is then injected into the sample compartment and reacts with the membrane.

MATERIALS AND METHODS

Extraction

Three grams of fish flesh were homogenized in ten ml of 10% trichloroacetic acid (TCA) using a glass mortar and pestle. After the solids settle, three ml of the liquid was filtered, placed in a test tube, diluted 2 fold with buffer and neutralized using 2M NaOH. A small portion (50 ul) of the neutralized sample was injected into the sample compartment. From the same sample tube, 500 ml was placed into the reaction tube containing immobilized enzyme and vortexed for 2 minutes. A small portion (50 ul) of the enzyme reacted sample was then injected into the instrument. The K value was digitally displayed on the front of the biosensor.

Methodology

Three different storage studies are presented in this paper. In the first study, 5 species of fish were obtained fresh and placed on ice in plastic containers with holes drilled in the bottom for drainage. The samples were stored at 4 degrees C for two weeks with the lids of each container open during the day and closed at night. The five species included catfish (Ictalurus punctatus), hybrid striped bass (a genetic cross between striped bass Morone saxatilis and white bass Morone chrysops), tilapia (Tilapia aurea), grouper (Mysteroberca bonaci) and salmon (Salmo salar). An initial (day 0) K-value was determined for each and then subsequent K value measurements were made every few days. The raw fillets were also organoleptically evaluated at each sample time. A second trial with grouper fillets involved storage as in the first trial. Again, K-values were obtained on day 0 and every few days following. Organoleptic evaluations were also made at each sampling time. The final trial with catfish fillets involved similar storage on ice in refrigeration for 2 weeks. Samples were taken daily and evaluated organoleptically when K-values were also measured.
Sensory Evaluations

Raw fillets in each of the three studies were evaluated on the same days that K-values were measured. The fish samples were rated on appearance, texture and odor by 5-10 panelist who were experienced in descriptive evaluation of fish and shrimp. The panelist were presented the raw fillets in the storage container and a rating scale of 1 to 10 was used where 1 was excellent and 10 unacceptable. The score of 5 was considered marginal. To determine the texture, the panelist used a fork to separate the meat and were allowed to handle a small portion of the fillets. Average scores for each fillet were calculated.

RESULTS

In the first study, initially all five species of fish were of exceptional quality, with K values less than 0.2 (Figure 1). On day 5, tilapia and salmon remained fresh (K-values<0.2, organoleptic rating<2) while the catfish, hybrid striped bass and grouper were beginning to show organoleptic signs of degradation. The edges of the fillets were drying and the overall raw muscle surface color yellow (K-values approximately 0.4). At day 7, tilapia and hybrid striped bass showed more severe signs of degradation having organoleptic ratings of 10 which corresponded to K-values slightly greater than 0.4, while the catfish, grouper and salmon were organoleptically rated 2-4. After 10 days, all fish species were showing organoleptic signs of spoilage and tilapia and hybrid striped bass scored higher than the other species (K-values 0.68-0.95). In most cases, the muscle fibers were gaping, the edges of the fillets were dry, the overall color of the fillets was dark yellow to brown and an ammonia, sour odor was detected (organoleptic ratings 6-10). Organoleptically, the catfish, grouper and salmon were rated from 4-7 which was slightly below marginal to slightly above marginal. The K values corresponded well with the organoleptic ratings (Figure 2). At the day 5 rating both the tilapia and the hybrid striped bass were above grade 5 on the sensory scale. On day 10 all the species were unacceptable except the salmon and the grouper. A similar trend was seen for the grouper storage study. The K value increased significantly between day 2 and 4 but remained the same until day 7 and then increased. As seen in Figure 3, the sensory ratings also increased steadily over time and the grouper became unacceptable at day 12 when the K values were very close to 1.0. In the third study, the catfish had very low K values until day 7 and then steadily increased (Figure 4). This also corresponded well with the sensory ratings. On day 7 the sensory evaluations were higher than the K values and the catfish was rated unacceptable on day 15 while the K values were still in the acceptable range.

CONCLUSIONS

The K-values obtained with the MICROFRESH Biosensor appear to correlate well with work found in the literature. This instrument provided an easy and reproducible means in which to measure K values for several species of fish. The K values obtained using the Biosensor from Pegasus Biotechnology also compare well with sensory ratings. Further analytical work involving variabie storage temperature, larger sample sizes and more fish species is necessary to confirm these results.
REFERENCES


FIGURE 1.  

**K VALUES**

![Graph showing K values for different fish species over days.]

**AVERAGE STANDARD DEVIATION WAS +/- 10%**.

FIGURE 2.  

**ORGANOLEPTIC EVALUATION**

![Graph showing organoleptic evaluation scores for different fish species over days.]

**1-10 SENSORY RATING SCALE**

1-EXCELLENT 5-MARGINAL 10-UNACCEPTABLE
Figure 3.

Storage Study

(Grouper)

K VALUES

SENSORY RATING

Average standard deviation for all analyses was +/- 0.05

Sensory scale 1-10: 1-Excellent 5-Marginal 10-Unacceptable

Figure 4.

Storage Study

(Catfish)

K VALUES

SENSORY RATING

Average standard deviation for all analyses was +/- 0.05

Sensory scale 1-10: 1-Excellent 5-Marginal 10-Unacceptable
SEASONAL VARIATIONS IN FATTY ACIDS OF 34 SPECIES OF FINFISH

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Consumers are becoming more aware of the importance of nutrition to human health, and with this interest the popularity of seafood has surged. Seafood contains n-3 or omega-3 fatty acids which may play a role in preventing and lessening the impact of certain diseases. The increased interest in seafood brings with it concern for consistency of seafood products. Any wild caught product is subject to a broad spectrum of variability factors, not the least of which is the variation in nutrient level imposed by seasonal influences.

As early as 1973, Stansby (11) observed strong seasonal variations in lipid content in fish. He concluded that fish that stored triglycerides in their muscle tissue showed maximum fat storage in the summer. However, Kinsella (5) in a review of seasonal variations observed that for some fishes the fat storage peaked in colder months. Other studies (2,3,4,8,9) also showed that there were significant seasonal fluctuations in lipid composition and that these variations were not consistent among species.

In a 3-year study of fatty acids and other nutritional components in Gulf of Mexico finfishes, an effort was made to collect 42 targeted species in all seasons. These collections were examined to define both the mean seasonal values of lipid and fatty acid concentrations and the ranges these values might encompass due to seasonal variability. In addition, these collections allowed assessment of seasonal availability of Gulf fishes as well as comparison of fatty acid levels and distributions to those in cold water and tropical fishes.

MATERIALS AND METHODS

Sample preparation
Immediately after delivery of fresh fish to the laboratory, fish were measured, weighed and state of health recorded. Fish were filleted and given identification number, placed in plastic containers, flushed with N₂ to remove O₂ and then frozen at -40°C. At the end of each season, the entire collection of each species of individual fishes were grouped into a minimum of three pools. Identical quantities of tissue from each of the fish in the subgroups were used in the composites so that no bias was placed upon size of fish. Tissues were taken from the entire longitudinal cross section of fillet so that the sample was representative of all of the edible tissue of each fish.

Analytical procedure
All solvents used in analysis were HPLC grade or analytical reagent grade. Standards were purchased from NuCheck Prep, Inc. (Elysian, MN). Fillets were homogenized using a Waring blender and 0.5 g aliquots weighed into screw-capped (Teflon-lined) centrifuge
tubes (30 ml) and saponified at ambient temperature with ethanolic KOH under N₂ using a magnetic stirrer for one hour. Care was exercised in the volumes of saponifying mixtures used to keep the water level, derived from tissue, sufficiently high to prevent transesterification. Solvent ratios were those suggested by Nelson (7). After dilution with distilled water, the neutral fraction was extracted with hexane. The remaining alkaline solution was acidified with 6N HCl, and free fatty acids were extracted with benzene. Benzene aliquots were combined and concentrated using a rotary evaporator. All evaporations were closely monitored to ensure that distillation temperatures did not exceed 25°C. Fatty acids were converted to methyl esters using 7% BF₃-MeOH by the method of Metcalfe et al. (6) modified to use ambient temperatures and a one-hour reaction period.

Identification of fatty acid methyl esters (FAME) was obtained by capillary gas chromatography (GC) using a Perkin-Elmer model Sigma 2000 gas chromatograph equipped with flame ionization detector and fitted with a 30 m x 0.25 mm i.d. fused silica capillary column coated with a 0.25 µm film thickness of Dura Bond WAX (J & W Scientific) and operated with a split ratio of 100:1. The carrier gas was He, maintained at 20 psi. Oven temperature was programmed at 90-250°C at a linear rate of 4°/min. Data was processed using a Perkin-Elmer Sigma 10 data system with quantification of all compounds based on individual peak area response by GC compared to the internal standard methyl tricosanoate. Quantitative data were corrected for differences in detector responses that were determined through analysis of authentic standards of each reported fatty acid. FAME were tentatively identified by comparison with retention times with those of authentic standards. Verification of identification on select samples was accomplished through gas chromatography mass spectrometry analysis conducted by Charleston Laboratory, National Marine Fisheries Service.

Sample protection
Several precautions were taken to ensure that no degradation or other alteration of lipids occurred during extraction and saponification. All analytical steps were conducted at ambient temperatures, and samples were constantly flushed with N₂ to prevent oxidation. Further, as many steps as possible were conducted in a single extraction tube to reduce loss and degradation that occurs with sample transfer. All solvents were flushed with N₂ immediately before use to remove dissolved O₂ and to prevent oxidative degradation. Likewise, samples requiring storage were placed in sample bags which were then flushed with N₂ before being placed in freezers. In addition, the antioxidant BHT was added in a concentration of 0.005% (w/v) to extraction solvents to prevent oxidative degradation of unsaturated lipids.

Data analysis
One way analysis of variance (ANOVA) with post facto 95% confidence level range test (12) was used to compare fatty acids as well as certain parameters derived from fatty acid data. Those parameters that indicated statistical difference within comparison groups were identified (p<0.05).

RESULTS AND DISCUSSION

Seasonal availability
Of 42 species of fish included in this study, 34 were collected in two or more seasons. Collections are listed in Table 1. Though many of these species are not typical of those found in seafood markets, all have been identified as having good market potential.
Table 1. Seasonal Collections of Gulf of Mexico Finfishes*

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*Collections within each season indicated by: Sp-spring, Su-summer, Fa-fall and Wi-winter; collections only listed for which analyses have been completed as of this report.

Total lipids and fatty acids

Much of the interest in seafood as a consumer product lies in the fact that fish is low in fat and cholesterol and is a good low-fat alternative to other protein sources. A fat level of <5% is generally considered to be "low fat". In this regard Gulf of Mexico fishes generally fall in the low fat category. Lipid and total fatty acid concentrations are displayed graphically in Figure 1 for those fishes collected and analyzed in three or four seasons. Both variables displayed similar seasonal trends, and using these trends the fishes fell into the two categories previously described by Shulman (10). One group which he called "heat loving" deposited fat in the summer and fall with sexual maturation occurring in spring and spawning in summer. The "cold loving" group deposited fat in the spring and summer, and sexual maturation occurred in the fall and spawning in winter. Bluefish, pigfish and spot fit the "heat-loving" description having greater fat in summer and fall while hardhead catfish, sheepshead and spotted seatrout fit the "cold-loving" description having fat storage in winter and spring.
N-3 fatty acids

The quantity of n-3 fatty acids in seafood is of particular interest to the astute seafood consumer because of the purported benefits associated with replacing some of the excessive quantities of n-6 fatty acids derived from beef, pork and poultry in the American diet with n-3 fatty acids from seafood. In Figure 2, n-3 fatty acid concentrations are shown both as absolute amounts of mg/g in fish tissue and as relative percent of total fatty acids. All fishes, regardless of season, showed appreciable levels of n-3 in edible tissue (1-14 mg/g) but with two contrasting seasonal variation patterns. In one pattern, bluefish, dolphin, hardhead catfish and red porgy displayed inverse trends in absolute and relative amounts of n-3 i.e. seasonal increases in absolute quantities of n-3 were accompanied by decreases in the relative percent of total fatty acids. These fish apparently accumulate n-3 fatty acids less readily than other fatty acids during periods of high fat deposition. Another group of fish, gulf butterfish, red drum, scaled sardine and sheepshead showed similar trends for both absolute and relative concentrations of n-3. This indicated that seasonal increases in absolute n-3 fatty acids were accompanied by increases in relative percent of total fatty acids. These observations most likely reflect particular dietary patterns of the various Gulf finfishes, with diet serving as the major influence on the n-3 fatty acid levels in fish tissue.
Figure 2. N-3 fatty acids in Gulf of Mexico fishes.

Specific n-3 and n-6 fatty acids
Attention has been focused on three polyunsaturated fatty acid components, the n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the n-6 fatty acid, arachidonic acid (AA). Excessive quantities of arachidonic acid and its precursors in the diet can lead to formation of certain eicosanoids that may produce undesirable health effects. These effects can be countered by addition of n-3, primarily EPA and DHA, to the diet. Figure 3 displays seasonal variations of the ratio of EPA+DHA to AA. The ratio was >1 regardless of species or season. Several of the fishes have maxima in this ratio in the winter or spring season duplicating seasonal trends seen in overall n-3 to n-6 ratios shown in Figure 4 and discussed below.

EPA is the primary n-3 component found in cold water fish, and for that reason has received considerable attention in research concerning the role of n-3 fatty acids in the diet. DHA has been reported as the predominant n-3 fatty acid in tropical fish (1) and has received much less attention in medical research. It is interesting that Evans (1) noted an increase in DHA/EPA ratios in fish from more tropical waters suggesting that DHA predominance is linked to geographical temperature zones. Our study showed DHA/EPA ratios >1 in Gulf fish with some interesting seasonal trends. Patterns of DHA/EPA seen in Figure 3 closely resembled those of n-3 (% fatty acids) shown in Figure 2. This relationship suggests that when fatty acids in Gulf fishes were most enriched in n-3 fatty acids that DHA also reached its maximum values. It is hypothesized that EPA may be the conservative component of the n-3 fatty acids with fluctuations in these n-3 fatty acids primarily attributable to variations in the levels of DHA.
Figure 3. Eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic acids (AA) in Gulf of Mexico fishes.

Figure 4. Ratios of n-3/n-6 and unsaturated/saturated fatty acids in Gulf of Mexico fishes.
**Ratios of n-3/n-6 and unsaturated/saturated**

In Figure 4 the ratios of total unsaturated fatty acids to saturated fatty acids are remarkable for their consistency not only across season but across species. Though some seasonal trends indicate statistically significant differences, absolute differences from a consumer perspective appear to be inconsequential. This ratio may be regarded as a conservative property of Gulf seafood regardless of season or species. The levels of total n-3 to total n-6, like the (EPA+DHA)/AA ratios, tended to maximize in winter and spring for many of the fishes. Using the criterion of enhanced n-3/n-6 or enhanced (EPA+DHA)/AA as a desirable trait of seafood, there may be some slight seasonal advantage to Gulf fish caught in either the winter or spring months.

**Interseasonal trends**

Another way of examining seasonal fatty acid data for multiple species is to examine one or two variables within one season for all fish species and see how trends among fishes differ between seasons. Such a treatment is shown in Figures 5-8 which are seasonal plots of n-3 and n-6 as % of fatty acids vs lipid as wt % of tissue. Individual data points represent mean values for one species during a season. Correlation coefficients (R) for all plots except the spring and fall n-6 fatty acids were significant at the p<0.05 level and indicated significant relationships between these pairs of variables. With leaner fish represented by points to the left and fattier fish to the right, it can be seen that n-6 levels did not differ appreciably between fattier and leaner fish during any season, and in the spring showed no distinction whatever. The negative slopes in all the n-3 plots indicated that as one progresses towards the leaner fish, one encounters fish with fatty acids progressively enriched in the n-3 fatty acids, an observation also noted previously (1). The slopes of -4.1 and -4.8 for winter and spring collections (vs -2.1 and -2.4 for summer and fall) indicated greater enrichment in n-3 fatty acids in leaner fish (compared to fattier fish) in the winter and spring months. This observation supports the earlier contention that Gulf fishes, particularly the leaner fishes, have some advantage in both quantities and balance of the n-3 fatty acids in the winter and spring months.

**Warm water vs cold water fishes**

Published literature extols the benefit of seafood consumption and often suggests that one consume cold water fish. Many cold water fishes have greater absolute quantities of n-3 in edible tissue than warm water fishes, but at the expense generally of higher total fats. This offers little if any advantage in terms of n-3/n-6 balance in the diet. Enhancement of n-3/n-6 in the diet is best accomplished by lowering the fat intake and enhancing the n-3/n-6 ratio rather than offsetting excessive n-6 fatty acid consumption with large quantities of additional fat, that is enriched in n-3 fatty acids. A low fat diet is compatible with the leaner Gulf of Mexico warm water fish, which coupled with other low fat foods, can effectively raise the n-3/n-6 ratio in the diet to more desirable levels.

**Conclusion**

Gulf of Mexico fishes are low in fat but both lipid and fatty acids have considerable variation during the year. The n-3 fatty acids are predominant over n-6 fats with somewhat higher ratios observed in the winter and spring seasons. Seasonal variations are the result of food availability as well as feeding and migratory patterns; this accounts for a large part of the diversity in seasonal patterns observed with warm water fishes. The choice of seafood need not be a choice of cold or warm water species or a particular species or one caught during a certain season, but should be a choice of a variety of whatever fresh fish is available in the region.
Figure 5. N-3 and n-6 fatty acids vs fats in winter collections. Each point represents mean for one fish species.

Figure 6. N-3 and n-6 fatty acids vs fats in spring collections. Each point represents mean for one fish species.
Figure 7. N-3 and n-6 fatty acids vs fats in summer collections. Each point represents mean for one fish species.

Figure 8. N-3 and n-6 fatty acids vs fats in fall collections. Each point represents mean for one fish species.
ACKNOWLEDGMENTS

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