PURIFICATION AND CHARACTERIZATION OF A PROTEASE WITH TRYPsic 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 hydrolyses. 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 CaCl₂, pH 7.8) in a ratio of 1:5 (w/v). The slurry was stirred for 4 hours at 4°C and centrifuged at 3000g for 30 min at 4°C. Brij 35 was added to the supernatant to a final concentration of 0.02%. The supernatant was stirred overnight at 4°C and finally centrifuged at 10,000g for 30 min at 4°C. The sediment was discarded, the supernatant collected was fractionated with solid ammonium sulfate at 4°C 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°C. Three volumes of cold acetone (-20°C) were added and the precipitate formed was collected by centrifugation at 6000g for 30 min at 4°C. The acetone precipitate was dried first with cold (-20°C) acetone-ether (1:1 v/v) and then cold (-20°C) peroxide-free ether. The dried material was suspended in a minimum amount of extraction buffer and centrifuged at 10,000g for 30 min at 4°C. The supernatant obtained was incubated at 4°C 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 Nα-Benzyoxarginine-p-nitroanilide (BAPA) as substrate. One BAPA unit of activity was defined as ΔA₄₀₃µm/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 ΔA₂₄₇µm/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).
Electrophoresis:

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 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.

<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</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Sup' 1</td>
<td>1295.00</td>
<td>19.60</td>
<td>0.0148</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>'Sup' 2</td>
<td>941.00</td>
<td>19.47</td>
<td>0.0207</td>
<td>101.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>88.11</td>
<td>18.27</td>
<td>0.2100</td>
<td>93.2</td>
<td>14.0</td>
</tr>
<tr>
<td>Acetone fraction</td>
<td>26.93</td>
<td>11.41</td>
<td>0.4237</td>
<td>59.6</td>
<td>29.0</td>
</tr>
<tr>
<td>Aminoben-zamidine fraction</td>
<td>3.77</td>
<td>4.26</td>
<td>1.1300</td>
<td>22.3</td>
<td>76.0</td>
</tr>
<tr>
<td>SBTI fraction</td>
<td>2.32</td>
<td>3.15</td>
<td>1.3560</td>
<td>16.4</td>
<td>92.0</td>
</tr>
</tbody>
</table>

The optimum pH for hydrolysis of BAPA at 25°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 56°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°C, and all of its activity at 75°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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>Concentration</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>TAME</td>
<td>5.00 mM</td>
<td>62</td>
</tr>
<tr>
<td>SBTI</td>
<td>BAPA</td>
<td>25.00 mM</td>
<td>93</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>TAME</td>
<td>0.25 (TIU/mL)</td>
<td>96</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>BAPA</td>
<td>2.50 mM</td>
<td>73</td>
</tr>
</tbody>
</table>

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.

REFERENCES


Overnall J. (1973) Digestive enzymes of the pyloric ceca and of their associated mesentery in the cod (Gadus morrhua). Comp. Biochem. Physiol. 46B, 519-531.


ISOLATION AND CHARACTERIZATION OF TWO TRYPsin-LIKE ENZYMES FROM MENHADEN, Brevoortia tyrannus, 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 tyrannus, 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 tyrannus, 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 CaCl₂ 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 CaCl₂ 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-Sephalcel 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 CaCl₂. 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°C.

**Effect of Inhibitors**

Tosyl-L-lysine chloromethyl ketone (TLCK, 2.5 µM), benzamidine (1.25 mM), diisoproporphosphate (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°C for 15 min. After incubation, residual trypsic activity was determined with BAPNA.

**RESULTS & DISCUSSIONS**

**Purification of Trypsins from Menhaden Intestine**

A chromatogram of a DEAE-Sephacon 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-Sephael 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

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

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 anhydrase.
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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc.</th>
<th>Enzyme A</th>
<th>Enzyme B</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Tosyl-L-lysine chloromethyl ketone</td>
<td>2.5 mM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tosyl-L-phenylalanine chloromethyl ketone</td>
<td>1.25 mM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>0.15 mg/ml</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diisofluorophosphate</td>
<td>1.0 mM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>1.25 mM</td>
<td>14</td>
<td>43</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td>2.0 mM</td>
<td>84</td>
<td>56</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetate</td>
<td>0.5 mM</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>2.15 μg/ml</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Antipain</td>
<td>8.50 μg/ml</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
REFERENCES


Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the


DEVELOPMENT OF ROCK SHRIMP SPECIFIC MONOClonAL ANTIBODY FOR ROCK SHRIMP
IDENTIFICATION

C.I. Wei1, H. An1, M.R. Marshall1, W. S. Otwell1, K.J. Kao2, and P.A. Klein2

Food Science and Human Nutrition Department1 and Department of Pathology and
Laboratory Medicine2, 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
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. Electrophoretic patterns were recorded by developing the positive image using a Kodak Electrophoretic 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 a-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 Na₂HPO₄ and 0.3 M KH₂PO₄. 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/O 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 membrane 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 MgCl₂ (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:

\[ \text{Rock shrimp content (ng)} = (\text{Protein content, ng}) \times (\text{Rock shrimp percentage}) \times (\text{Dilution factor}) \]

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 Rv 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
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Z1</td>
<td>50% TR + 50% SS</td>
</tr>
<tr>
<td>Z2</td>
<td>50% S2 + 50% B</td>
</tr>
<tr>
<td>Z3</td>
<td>50% S + 50% F</td>
</tr>
<tr>
<td>Z4</td>
<td>50% R2 + 50% PBS</td>
</tr>
<tr>
<td>Z5</td>
<td>50% R1 + 50% PBS</td>
</tr>
<tr>
<td>Z6</td>
<td>50% S7 + 50% S9</td>
</tr>
<tr>
<td>Z7</td>
<td>50% O + 50% TU</td>
</tr>
<tr>
<td>Z8</td>
<td>50% S10 + 50% L1</td>
</tr>
<tr>
<td>Z9</td>
<td>50% S5 + 50% C</td>
</tr>
<tr>
<td>Z10</td>
<td>50% G + 50% P</td>
</tr>
<tr>
<td>Z11</td>
<td>50% S3 + 50% L2</td>
</tr>
<tr>
<td>Z12</td>
<td>50% S8 + 50% S4</td>
</tr>
<tr>
<td>Z13</td>
<td>50% R3 + 50% PBS</td>
</tr>
<tr>
<td>Z14</td>
<td>33% CH + 33% S1 + 33% S6</td>
</tr>
<tr>
<td>Z15</td>
<td>33% R2 + 33% O + 33% SS</td>
</tr>
<tr>
<td>Z16</td>
<td>33% R2 + 33% C + 33% S10</td>
</tr>
<tr>
<td>Z17</td>
<td>33% R3 + 33% S4 + 33% S1</td>
</tr>
<tr>
<td>Z18</td>
<td>33% R3 + 33% CH + 33% L2</td>
</tr>
<tr>
<td>Z19</td>
<td>33% R1 + 33% S10 + 33% L1</td>
</tr>
<tr>
<td>Z20</td>
<td>17% R1 + 17% R2 + 17% R3 + 33% G + 17% PBS</td>
</tr>
<tr>
<td>Z21</td>
<td>17% TR + 17% SS + 17% S2 + 17% B + 33% R2</td>
</tr>
<tr>
<td>Z22</td>
<td>17% TR + 17% S + 17% F + 17% R1 + 17% S7 + 17% S9</td>
</tr>
<tr>
<td>Z23</td>
<td>17% O + 17% SS + 17% C + 17% S10 + 33% R2</td>
</tr>
<tr>
<td>Z24</td>
<td>29% R1 + 14% S5 + 29% P + 29% PBS</td>
</tr>
</tbody>
</table>

*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 Argentina; 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 Canavare, 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 (Foegeing, 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 IgG1(k) isotype as determined by the isotyping kit (data not shown). In the presence of β-mercaptoethanol, 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 Canaveral, 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 Port 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 4H2-1OD3 McAb\textsuperscript{a} with the water extracts\textsuperscript{b} of the various seafood and meat samples in the blind study using the ELISA test.

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

\textsuperscript{a} The McAb used was 10 µg protein/well.

\textsuperscript{b} The protein extracts used was 1 µg/well.

\textsuperscript{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.

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

* Positive mixture samples containing rock shrimp as determined in the blind study by ELISA.

* Rock shrimp content was calculated by multiplying protein content with the dilution range and rock shrimp percentage in the mixture.
CONCLUSION

In summary, the 4H2-1OD3 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

This article was developed under the auspices of the Florida Sea Grant College Program with support from the National Oceanic and Atmospheric Administration, Office of Sea Grant, U.S. Dept. of Commerce, Grant No. R/LR-Q-11-PD.

REFERENCES


