Purification and Characterization of Alkaline Proteinase from Atlantic Menhaden Muscle


ABSTRACT

Two alkaline proteinases (A and B) were isolated and found to be composed of homogeneous subunits. These proteinases, A and B, were concentrated 62.9- and 986.5-fold compared to the crude muscle extract, with molecular weights of 707,000 and 450,000, respectively. Both are probably serine type proteinases, and optimum caseinolytic activity was shown at pH 8.0 and 55 °C. Both degraded actomyosin under similar conditions. Enzyme A had higher thermal stability than B. The residual activities of A and B in 3.6% NaCl solution were 95% and 85% and optimum caseinolytic activity was shown at pH 8.0 and 55 °C. Both degraded 707,000 and 450,000, respectively. Both are probably serine type proteinases, homogeneous subunits. These proteinases, A and B, were concentrated 62.9- and 986.5-fold compared to the crude muscle extract, with molecular weights of 707,000 and 450,000, respectively. Both are probably serine type proteinases, and optimum caseinolytic activity was shown at pH 8.0 and 55 °C. Both degraded actomyosin under similar conditions. Enzyme A had higher thermal stability than B. The residual activities of A and B in 3.6% NaCl solution were 95% and 85%. These data suggest that these proteinases are involved in the softening of menhaden surimi gels which occurs during heating at 50 to 70 °C.

Key Words: Atlantic menhaden, alkaline proteinase, gel strength, serine proteinase

INTRODUCTION

THE MENHADEN RESOURCE OF THE SOUTH Atlantic and Gulf of Mexico in the United States has not been used for surimi production because it is a fatty, dark-fleshed fish species, unlike the leaner, white-fleshed species now preferred for surimi manufacture. However, processing techniques are available that can recover a potentially marketable surimi or surimi-like product from pelagic species (Lanier, 1988, 1994), and there may be more use of such species as stocks of white-fish species become depleted. However, muscles of such species have higher levels of endogenous heat-stable proteinases that cause poor gel-forming properties during heating (Lanier, 1988).

Fish skeletal muscle contains many different proteinases. Cathepsin D from tilapia (Doke et al., 1980), cathepsins B and L from chum salmon (Yamashita and Konagaya, 1990, 1991), and heat-stable alkaline proteinase from carp (Makinodan and Ikeda, 1969; Iwata et al., 1973) and white croaker (Iwata et al., 1974) have been purified and examined for enzymatic properties. Heat-stable alkaline proteinase has been classified as a cysteine proteinase (Makinodan and Ikeda, 1971; Lin and Lanier, 1980).

A decrease in strength of gels from minced fish muscle can occur rapidly at 60 °C. Heat-stable proteinase has been implicated as the main factor causing this heat-induced softening (Makinodan and Ikeda, 1969, 1971), though Iwata et al. (1974) concluded that muscle alkaline proteinase did not take part in heat-induced softening. Su et al. (1981a) established a correlation between alkaline proteolytic activity, myosin degradation, and softening in fish muscle gels cooked at 60 °C.

Toyohara and Shimizu (1988) concluded that heat-induced softening in threadfin bream muscle could partly be due to the breakdown of myosin heavy chain (MHC) caused by an endogenous serine proteinase. Yanagihara et al. (1991) suggested that serine proteinase extracted from Atlantic croaker and threadfin bream muscle caused the degradation of MHC in fish meat gels induced by heating at 65 °C. They determined serine proteinase activity in various fish muscles asayed for enzyme activity above 50 °C.

The objective of this study was to investigate the properties of purified alkaline proteinase from Atlantic menhaden muscle, including its ability to degrade actomyosin.

MATERIALS & METHODS

ATLANTIC MENHADEN, BREVOORTIA TYRANNUS, (370 g and 27-cm body length on average) were purchased at the North Carolina coast in a very fresh state (pound or gill net caught) and immediately transported to the laboratory on ice. Excised muscle was minced and homogenized with 2 volumes of distilled water in a Sorvall Omni-mixer in an appropriate amount of 20 mM Tris-HCl, pH 8.0, and dialyzed against the same buffer overnight. The dialysates were applied to a DEAE Sephadex column (2.6 cm × 60 cm) equilibrated with the same buffer. The column was eluted with a 2000 mL linear gradient ranging from 0 to 2 M NaCl in 20 mM Tris-HCl, pH 8.0. Two proteinase fractions (A and B) were separated, and each fraction was dialyzed against 20 mM Tris-HCl, pH 8.0. Each dialysate was applied to the second DEAE-Sepharose column (2.6 cm × 60 cm) and eluted with a 1000 mL linear gradient ranging from 0 to 0.5 M NaCl. The caseinolytic active fractions were pooled and concentrated with ultrafiltration in an Amicon stirred cell (Amicon Instrument, Lexington, Mass., U.S.A.) using a PM 10 membrane. The concentrated samples were applied to a Sephacryl S-300 column (1.6 cm × 75 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and eluted with the same buffer. The purified proteinases A and B were concentrated and dialyzed against 20 mM Tris-HCl, pH 8.0. Protein concentration was measured at 280 nm, and proteinase activity for casein was measured at 0.1 M Tris-HCl, pH 8.0 and 40 °C during purification. The final products were stored at -20 °C and used for further studies.

Preparation of actomyosin

Actomyosin was prepared from Atlantic menhaden muscle according to the method of Nam et al. (1984). The minced muscle was washed four times with 20 mM sodium phosphate, pH 7.5, to remove sarcoplasmic protein and centrifuged after each wash at 3,000 × g for 10 min. The crude actomyosin was extracted from the precipitates with 20 mM sodium phosphate, pH 7.5, containing 0.45M KCl, for 20 h after final centrifugation for removing sarcoplasmic protein, and centrifuged at 8,000 × g for 15 min. The supernatant was filtered with two layers of gauze to remove insoluble materials. Filtrates were added to 10 volumes of cold water. After the
actomyosin settled, it was centrifuged at 6,000 × g for 10 min, and the same procedure was repeated 3 times to refine the actomyosin. The precipitates were dissolved in 0.6 M KCl-0.04 M Tris-HCl, pH 7.0, and dialyzed against the same buffer. The dialysates were centrifuged at 20,000 × g for 60 min to remove denatured actomyosin and the supernatant used as purified actomyosin.

Assay of proteolytic activity

Caseinolytic activity was determined throughout the purification process according to the method of Pyeon and Kim (1986). The assay mixture was composed of 0.75 mL buffer solution, 0.25 mL 2.5% casein solution, and 50 µL enzyme solution. The reaction was stopped by adding 0.5 mL of 16% (w/v) trichloroacetic acid (TCA) solution after 60 min incubation at the indicated temperature. After standing for 30 min at room temperature, the solution was centrifuged at 10,000 rpm for 10 min in an Eppendorf centrifuge, and 0.5 mL of the supernatant was assayed for TCA-soluble peptides according to the method of Lowry et al. (1951), calibrated with tyrosine. Blanks were prepared by adding TCA solution to the substrate prior to addition of enzyme. Units of caseinolytic activity were defined as µM tyrosine liberated/min/mL reaction mixture. Units of specific activity were defined as µM tyrosine liberated/min/mg protein. Chymotrypsin activity was determined by measuring the change in absorbance at 256 nm for the hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE). Trypsin activity was estimated by the change in absorbance at 247 nm for the p-tosyl-L-arginine methyl ester (TAME) (Hummel, 1959). The volume activities of BTEE and TAME hydrolyses were calculated as 31,100 × dA/min and 55,600 × dA/min, (dA is differential absorbance). Activity is expressed as units/L.

Optimum pH and temperature

The buffer solution was prepared according to the method of Dawson et al. (1969). Optimum pH for the proteolytic activity was measured at 55 °C and over the pH range of 6.0 to 10.5 (0.1 sodium phosphate for pH 6.0 to 7.2, 0.1 M Tris-HCl for pH 7.0 to 8.9, and 0.1 M sodium carbonate-bicarbonate for 9.0 to 10.5). The optimum temperature was measured at pH 8.0 for proteinases A and B over a range of 35 to 70 °C. Slight changes in pH with increasing temperature were not considered.

Protein concentration and electrophoresis

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) with 12% polyacrylamide gels at pH 8.0.

### Table 1—Purification of alkaline proteinase from Atlantic menhaden muscle

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Proteinase A</th>
<th>Proteinase B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activitya</td>
<td>Yield %</td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.03</td>
<td>100.0</td>
</tr>
<tr>
<td>Ammonium sulfate (20% to 70%)</td>
<td>0.06</td>
<td>93.3</td>
</tr>
<tr>
<td>1st DEAE-Sephalac</td>
<td>0.21</td>
<td>85.1</td>
</tr>
<tr>
<td>2nd DEAE-Sephalac</td>
<td>0.22</td>
<td>86.5</td>
</tr>
<tr>
<td>1st Sephacry S-300</td>
<td>0.30</td>
<td>12.4</td>
</tr>
<tr>
<td>2nd Sephacry S-300</td>
<td>1.63</td>
<td>2.8</td>
</tr>
</tbody>
</table>

aUnits are µM Tyr/min/mg-protein. Proteinolytic activities were measured at 0.1 M Tris-HCl, pH 8.0 and 40 °C.

Determination of molecular weight

The molecular weights of proteinases A and B were determined using a Sephacryl S-300 gel filtration column (1.6 cm × 75 cm) according to the method of Whitaker (1963) with molecular weight markers (Sigma, MW-GF-1000).

Effect of NaCl and thermal stability

The effect of NaCl concentration on caseinolytic activity was determined using an assay buffer containing NaCl at concentrations from 0.71% to 4.23% plus a control. The thermal stabilities of proteinases were measured as caseinolytic activity remaining at optimum pH and temperature after incubating 10 min at the indicated temperature.

Effects of inhibitors on proteinase activity

Enzyme solution (25 µL) was incubated with equal volumes of each inhibitor solution for 20 min at 30 °C. After incubation, remaining activities were assayed with 2.5% casein as substrate at pH 8.0 and 55 °C. Disopropyl fluorophosphate (DFP; 0.04 mM), 0.2 mM p-toluenesulfonyl-L-lysine chloromethyl ketone (TLCK), 2 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM 2-mercaptoethanol were dissolved in distilled water and used as inhibitors of proteinase activity. p-Toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK) was dissolved in dimethyl sulfoxide and diluted with distilled water to 0.2 mM concentration.

### Statistical analysis

Statistical analysis of data and simple linear regression was carried out using JMP 3.0 (SAS Institute Inc., Cary, N.C., U.S.A.).

### RESULTS & DISCUSSION

Purification of alkaline proteinases A and B

The specific activity and recovery of alkaline proteinases A and B during purification were summarized (Table 1). Proteinase A was purified 62.9-fold with a 2.8% yield, and proteinase B was purified 986.5-fold with a 6.3% yield. Comparison of the electrophoretic pattern of the crude extract with that of the solution after ammonium sulfate fractionation and the first DEAE-Sephalac ion exchange indicated that a considerable amount of low molecular weight and non-proteolytic materials were efficiently removed in the initial purification step. The second DEAE-Sephalac column separated two highly active peaks of alkaline proteinase (Fig. 1). The remaining purification steps were applied to remove contaminating proteins. Purity of the final products obtained by Sephacryl S-300 gel chromatography was established by SDS-PAGE at pH 8.0, where a single band was evidence of homogeneity (Fig. 2). Proteinases A and B did not bind with benzamidine-Sepharose 6B resin and the purity of enzyme was not increased by
Alkaline Proteinase of Menhaden Muscle...

Determination of molecular weight

Molecular weights of proteinases A and B were estimated to be 707,000 and 450,000 daltons, respectively (Fig. 3). These are similar to that of a serine type proteinase reported by Iwata et al. (1973) and Kinoshita et al. (1992). Molecular weights of trypsin-like A and B proteinases from Atlantic menhaden intestine as measured by gel chromatography were estimated to be 25,000 and 26,000 daltons (Pyueh et al., 1990). Molecular weights estimated by gel chromatography were 780,000 and 500,000 daltons, respectively, for alkaline proteinases from carp muscle (Iwata et al., 1973) and threadfin bream muscle (Kinoshita et al., 1992). The molecular weight of a serine proteinase from white croaker muscle was estimated to be about 680,000 by gel chromatography and about 32,000 and 71,000 by SDS-PAGE with or without reducing reagent, respectively (Yanagihara et al., 1991). Using the sedimentation equilibrium method, the molecular weight of serine proteinase was estimated to be 920,000 for white croaker muscle and 780,000 for barracuda muscle (Iwata et al., 1974). Molecular weight of cathepsin L for chum salmon muscle was estimated to be 28,000 daltons (Yamashita and Konagaya, 1990) and 25,800 daltons (Yamashita and Konagaya, 1974) for Atlantic menhaden actomyosin by proteinase B was evident at pH 7.5 and 55 °C, with measured activities of 0.48 µM Tyr/min/mg-protein.

Optimal conditions for casein hydrolysis were similar to those reported for Atlantic croaker (Su et al., 1981a), sardine (Makino-dan et al., 1983), white croaker (Iwata et al., 1974; Yanagihara, 1991), and Pacific whiting (Chang-Lee et al., 1989). The specific activities of proteinases A and B at optimum conditions were lower than that of the trypsin-like enzymes of Atlantic menhaden intestine (Pyueh et al., 1990). However, the results suggest alkaline proteinases A and B of Atlantic menhaden muscle are responsible for the degradation of its myofibrillar protein during heating.

Substrate specificity

The esterolytic activities of proteinases A and B were based on the hydrolysis of BTEE and TAME (Table 2). Proteinase A had nearly 7 times higher activity in the hydrolysis of BTEE than B. Proteinase B had no activity toward TAME, which is a specific substrate for trypsin. This indicates that proteinase B is a serine type enzyme. The hydrolysis rates of proteinase A toward synthetic substrates were higher than those of B, but specific activities of A with casein and actomyosin were lower than those of B. This suggests that proteinase B would be more important than proteinase A in the degradation of actomyosin during fish gel processing.

NaCl tolerance and thermal stability

The caseinolytic activities of proteinase B up to 2.8% NaCl concentration were higher than that of the control, while those of A decreased with increasing concentrations of NaCl (Fig. 6). Yet both proteinases could degrade actomyosin solubilized in 4.23% NaCl. This suggests that these proteinases would be responsible for the degradation of fish gels during heating, as surimi-based gel...
products typically contain 2% to 3% NaCl. A heat-stable proteinase from threadfin bream has been shown to have MHC-degrading activity in the presence of 3% NaCl (Kinoshita et al., 1990). They reported that, by solubilizing intact myofibrils, hydrolysis at the higher salt levels was enhanced. Cathepsin L from anchovy exhibited 67% remaining activity toward yellowtail myofibrillar protein in the presence of 5% NaCl (Heu, 1993).

After proteinases A and B in 20 mM Tris-HCl, pH 8.0, were incubated for 10 min at the indicated temperatures, residual activities with casein as substrate were determined at optimum pH and temperature (Fig. 7). Proteinase A was inactivated faster than B.

Remaining activities of proteinases A and B after denaturing at 55 °C for 10 min were 38% and 25%, respectively. The thermostabilities of proteinases A and B are similar to that of alkaline proteinase from white croaker (Iwata et al., 1974) but greater than a trypsin-like enzyme from menhaden intestine (Pyeun et al., 1990). The activity of similar proteinase extracted from Atlantic croaker skin and muscle remained fairly stable when preincubated at 60 °C for 20 min but was completely lost during preincubation at 70 °C (Su et al., 1981a). Activity of cathepsin L from anchovy remaining after preincubation at 50 °C for 30 min was 50% (Heu, 1993).

Effects of inhibitors

Proteinases A and B were inhibited by 0.04 mM DFP and 0.2 mM TPCK, specific inhibitors for chymotrypsin A (Zollner, 1989) but were not inhibited by 2 mM EDTA and 2 mM 2-mercaptoethanol (Table 3). From these results, we assumed that proteinases A and B were serine proteinases (chymotrypsin-like). Heat-stable proteinase from white croaker skeletal muscle was inhibited by DFP, soybean trypsin inhibitor, TLCK, and leupeptin (Yanagihara, et al., 1991). The proteinase from threadfin bream muscle was classified as a serine proteinase based on response to inhibitors (Kinoshita et al., 1990).

Table 3—Effect of inhibitors on the proteolytic activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (mM)</th>
<th>Proteinase</th>
<th>Proteinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.00</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>2-Mercapto-ethanol</td>
<td>2.00</td>
<td>95</td>
<td>92</td>
</tr>
<tr>
<td>TLCK</td>
<td>0.20</td>
<td>81</td>
<td>69</td>
</tr>
<tr>
<td>TPCK</td>
<td>0.20</td>
<td>54</td>
<td>49</td>
</tr>
<tr>
<td>DFP</td>
<td>0.04</td>
<td>7</td>
<td>12</td>
</tr>
</tbody>
</table>

EDTA—Ethylendiaminetetraacetic acid; TLCK-p-Toluenesulfonyl-L-lysine chloromethyl ketone; TPCK-p-Toluenesulfonyl-L-phenylalanine chloromethyl ketone; DFP—Dipropylfluorophosphate.

REFERENCES


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