Cathepsin L: A Predominant Heat-Activated Proteinase in Arrowtooth Flounder Muscle

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Characterization of the autolytic profile of arrowtooth flounder (ATF) muscle indicated the involvement of heat-activated proteinases active at both acidic and alkaline pH values. Further assay of fish extract exhibited the maximum activity at 60 °C against casein used as a substrate at both pH 5.5 and 8.0. The maximum activity shifted to lower temperatures by the addition of urea with two distinctive patterns: activity reduction at pH 5.5 and activity enhancement at pH 8.0. The highest inhibition by E-64 indicated the proteinase belongs to the cysteine proteinase class. At pH 5.5, the proteinase hydrolyzed Z-Phe-Arg-NMec and all types of protein substrates tested at higher rate than that at pH 8.0. Activity bands, observed on the activity-stained substrate gels, indicated similar proteinases are responsible for the proteolytic activity observed at both pH values. When proteins of fish extract were separated by HPLC-SEC, only one proteolytic peak was observed at the retention time of 26 min with an estimated molecular weight of 39800 Da. The results implied cathepsin L is a predominant proteinase responsible for autolysis of ATF muscle at elevated temperatures.

Keywords: *Cathepsin L; proteinase; fish; arrowtooth flounder*

INTRODUCTION

Arrowtooth flounder (ATF), *Atherestes stomias*, is the most abundant species in the Gulf of Alaska along the Aleutian chain and in the Bering Sea and is found on the Pacific coast of the United States and Canada (*1*). Arrowtooth flounder has been underutilized due to the problem of muscle softening during processing and cooking, resulting from the endogenous protease activity in the muscle (*2*). A number of endogenous proteases have been investigated as enzymes contributing to postmortem softening of fish flesh, which also participate to a different extent in the degradation of myofibrillar proteins (*3*−*5*). The action of these enzymes exerts detrimental effects on the sensory quality and functional properties of muscle foods (*6*). Among them, heat-activated proteinases are of great concern for the utilization of fish because they are active at cooking temperatures and at neutral pH. In addition, they can damage fish textures and reduce the gel strength of surimi.

Proteolytic degradation of fish muscle proteins at elevated temperatures has been related to the presence of lysosomal cathepsins or alkaline proteinases. Many cathepsins have acidic pH optima, although some are most active at neutral pH (*7*). The cysteine-type cathepsins have a strong potential to be active at the postmortem muscle pH (5.5−6) and retain a significant level of activity up to pH 7 (*8*). Cathepsins B, H, and L have been found to cause softening of chum salmon (*9*), tilapia (*10*), and mackerel (*11*). Cathepsin L was a major proteinase found to degrade the myofibrillar proteins in Pacific whiting (*12*). The gel-softening phenomenon or "modori" observed at 50−70 °C was attributed to myosin hydrolysis by heat stable alkaline proteinases (*13*, *14*). Alkaline proteases isolated from fish muscle show wide variations in their characteristics. They are oligomeric protease with high molecular weights ranging from 560 to 920 kDa (*3*). However, an alkaline protease isolated by Lin and Lanier (*13*) from Atlantic croaker was a much smaller protein with molecular weight of 80−84 kDa. These enzymes normally exhibit little or no catalytic activity unless assayed at a nonphysiologically high temperature or activated by protein denaturing agents such as urea, fatty acids, or detergents (*15*).

The presence of a heat stable cysteine protease in ATF was first reported by Greene and Babbitt (*2*) and later partially characterized by Wasson et al. (*16*). The partially purified enzyme was shown to have an approximate molecular weight of 32000 Da and an optimal condition at pH 6.0−7.0 and 55 °C against casein. However, the identity of the active enzyme could not be clearly defined. Therefore, the objective of this study was to identify and study the hydrolytic properties of the major proteinase present in the muscle of ATF with a focus on the heat-activated proteinases involved in the autolysis of fish muscle at elevated temperature.

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MATERIALS AND METHODS

Chemicals. *N*-Carbobenzoxyphenylalanine-arginine 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), *N*-carbobenzoxyarginine-arginine 7-amido-4-methylcoumarin (Z-Arg-Arg-NMec), L-arginine 7-amido-4-methylcoumarin (L-Arg-NMec), 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), pepstatin, leupeptin, phenanthroline, ethylenediaminetetraacetic acid (EDTA), cytochrome C (horse heart), carbonic anhydrase, bovine serum albumin (BSA), hide powder azure, sweet potato amylase, blue dextran, Tris/base, 2-mercaptoethanol (βME), *p*-chloromercuribenzoate, aprotinin, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium caseinate was obtained from United States Biochemical Corp. (Cleveland, OH). Iodoacetic acid and phenylmethanesulfonyl fluoride (PMSF) were purchased from Calbiochem Co. (La Jolla, CA).

Samples. Arrowtooth flounders harvested off the Oregon Coast were obtained from a local processor as frozen fillets and kept frozen at −20 °C until used.

Autolytic Assays. Autolytic activity was measured according to the modified method of Greene and Babbitt (*2*). Frozen fish fillets were thawed and finely chopped. Three grams of chopped muscle was incubated for 30 min in a water bath at various temperatures, and the autolytic reaction was stopped by adding 27 mL of ice-cold 5% (w/v) trichloroacetic acid (TCA). To construct a pH profile, 3 g of chopped muscle was added with 12 mL of buffer (McIlvaine's buffer consisting of 0.2 M sodium phosphate and 0.1 M citric acid was used for the pH range of 2.5−8.0, and 0.2 M Tris buffer was used for the pH range of 8.5−9.0) and incubated for 30 min at 60 °C in a water bath; the reaction was stopped by adding 15 mL of ice-cold 9% (w/v) TCA. The mixtures were then homogenized for 1 min using a Polytron (Brinkmann Instruments, Westbury, NY) and kept on ice for 1 h followed by centrifugation at 5000*g* for 10 min to collect the TCA-soluble supernatant. Oligopeptide content was determined according to the Lowry assay (*17*), and autolytic activity was expressed as micromoles of tyrosine released per minute (μmol of Tyr/min).

Preparation of Fish Extract. Sarcoplasmic fluid of fish muscle was prepared according to the method of Seymour et al. (*18*). Frozen fish fillets were thawed, finely chopped, and then centrifuged at 5000*g* for 30 min to collect the supernatant as an enzyme source.

Enzyme Assay. Protease activity was assayed using casein as a substrate according to the method of An et al. (*12*). Activity was determined according to the TCA−Lowry assay. Fish extract (50−100 μL) was added to the preincubated reaction mixture of 2 mg of casein and 625 μL of McIlvaine's buffer at pH 5.5 or 8.0. Distilled water was added to bring the mixture to a final volume of 1.25 mL. The mixture was incubated at temperatures specified in the text for precisely 20 min. Enzymatic reaction was stopped by adding 200 μL of 50% (w/v) TCA. The reaction mixture was incubated at 4 °C for 15 min to precipitate unhydrolyzed proteins, and the mixture was then centrifuged at 5700*g* for 10 min to collect the supernatant. Activity was expressed as tyrosine equivalents in TCA supernatant, as measured by the Lowry assay (*17*). One unit of activity was defined as 1 nmol of tyrosine released per minute (nmol of Tyr/min). A blank was run in the same manner except the enzyme was added after the addition of TCA solution.

Effect of Urea. Effect of urea was tested by changes in the caseinolytic activity of fish extract in the presence and absence of urea in the reaction mixture. To minimize possible pH modification by addition of urea to the reaction mixture, urea was directly dissolved in McIlvaine's buffer at 5.0 and 8.0 M to give final concentrations of 2.5 and 4.0 M in the reaction mixture, respectively, and then the pH of the buffer was adjusted to pH 5.5 or 8.0. The enzyme activity was determined according to the TCA−Lowry assay as described above.

Effect of Inhibitors. Fish extract was incubated for 15 min at room temperature with an equal volume of 0.2 mM E-64, 2 mM sodium iodoacetate, 2 mM leupeptin, 20 mM sodium bisulfite, 2 mM PMSF, 2 mM 1,10-phenanthroline, 0.2 g/L soybean trypsin inhibitor, 2 mM PCMB, 4 μg/mL aprotinin, 0.2 mg/L pepstatin, 4 mM DTT, and 4 mM EDTA to give the final concentrations listed in Table 1. The residual activity was analyzed according to the TCA−Lowry assay using casein as a substrate. The initial stocks of E-64 and PMSF were prepared in DMSO and ethanol, respectively. Others were prepared in water.

Table 1. Effect of Proteinase Inhibitors on Proteinase in Arrowtooth Flounder Extract

inhibitor	concentration	% relative activity[a] pH 5.5	% relative activity[a] pH 8.0
E-64	0.1 mM	8.0	10.7
iodoacetate	1 mM	10.2	26.1
leupeptin	1 mM	12.1	15.4
sodium bisulfite	10 mM	47.9	93.5
PMSF	1 mM	73.7	55.9
1,10-phenanthroline	1 mM	83.2	87.9
soybean trypsin inhibitor	0.1 g/L	92.0	99.7
PCMB	0.1 mM	93.2	103.5
aprotinin	2 μg/mL	95.9	94.6
pepstatin	1 mg/L	97.1	97.4
DTT + EDTA	2 mM each	98.7	101.5

[a] Averages of duplicate analyses representing residual activity analyzed using casein as a substrate for 20 min under the specified conditions.

Hydrolysis of Synthetic Substrates. The hydrolytic activities on various synthetic substrates including Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and L-Arg-NMec were determined according to the method of Yamashita and Konagaya (*19*) with slight modification. Fish extract, 20−25 μL, was diluted to 1 mL with 0.1% Brij 35 and added with 500 μL of McIlvaine's buffer, pH 5.5 or 8.0, containing 8 mM DTT. To initiate the enzymatic reaction, 500 μL of 20 μM substrate solution was added to the mixture and then incubated at 55 °C for 10 min. The reaction was stopped by adding 200 μL of 5 mM iodoacetic acid. The fluorescence intensity of aminomethylcoumarin was determined at the excitation wavelength, 370 nm, and emission wavelength, 460 nm, using a luminescence spectrophotometer (LS 50B, Perkin-Elmer Ltd., Beaconsfield, U.K.). A unit of activity was expressed as 1 nmol of methylcoumarin released/min.

Effect of Neutral pH Incubation. Fish extract was incubated at 37 °C for 1, 2, 4, and 6 h with 10 volumes of 150 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 2 mM DTT, and 0.01% Triton X-100. The hydrolytic activity of preincubated fish extract was determined against Z-Phe-Arg-NMec according to the method previously described.

Hydrolysis of Protein Substrates. The protein substrates, that is, BSA, acid-denatured bovine hemoglobin, and purified ATF myofibrils (*20*), were tested as substrates in place of casein in the TCA−Lowry assay. One unit of activity is defined as nanomoles of tyrosine released per minute per milligram of protein (nmol of Tyr/mg/min). Hydrolytic activity against azocasein was performed according to the TCA−azocasein method (*12*) and expressed as A_{450}. Hide powder azure was tested by using the method of Rinderknecht et al. (*21*) with the slight modification of adding cold 50% (v/w) TCA solution to stop the reaction. Activity was determined spectrophotometrically at 595 nm and expressed as A_{595} compared with the absorbance of a blank.

Native Discontinuous Electrophoresis and Activity Staining. Native discontinuous electrophoresis was carried out by leaving out the SDS and reducing agent from the standard Laemmli protocol (*22*) and stained for protease activity with the modified method of García-Carreño et al. (*23*). Polyacrylamide gels were prepared for 5, 7.5, 10, and 12.5% running gels with 4% stacking gels. Fish extract was mixed with the sample treatment buffer [0.125 M Tris-HCl, pH 6.8, containing 20% (v/v) glycerol] at 1:1 (v/v) ratio, and 18−36 μg of proteins was loaded on the gel. The proteins were subjected to electrophoresis at a constant voltage of 75 V by mini Protean II (Bio-Rad Laboratories Inc., Richmond, CA). After electrophoresis, gels were immersed in 100 mL of 2% (w/v) casein in 50 mM Tris buffer, pH 7.5, for 1 h at 0 °C to allow the substrate

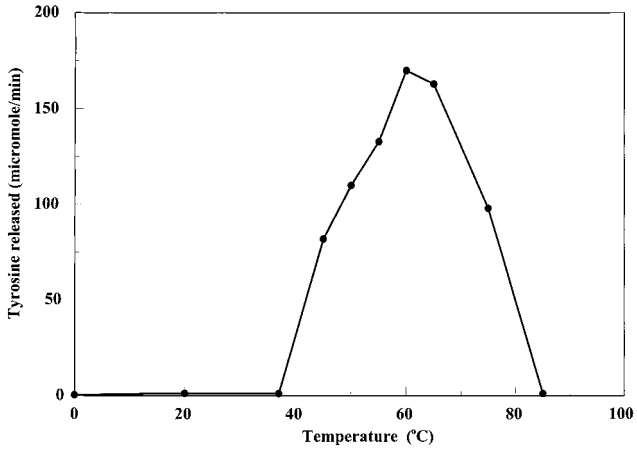

Figure 1. Temperature profile of ATF autolytic activity. Autolytic activity was determined by incubating ATF mince at various temperatures. The TCA-soluble proteins were recovered, determined by Lowry assay, and expressed as micromoles of tyrosine released per milliliter (μmol of Tyr/min).

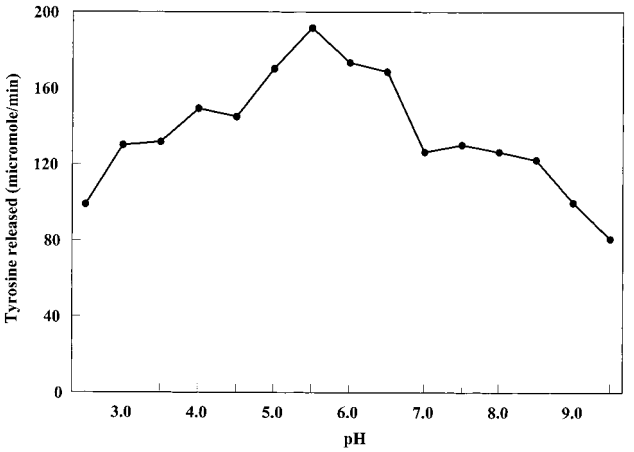

Figure 2. pH profiles of ATF autolytic activity. Autolytic activity was determined by incubating ATF mince at 60 °C at various pH values. The TCA-soluble proteins were recovered and determined by Lowry assay. The activity was expressed by change in liberated tyrosine (μmol Tyr/min).

to penetrate into the gels at reduced enzyme activity. The gels were then immersed at 60 °C for 1 h in 2% (w/v) casein (w/v) dissolved in McIlvaine's buffers, pH 5.5 and 8.0, containing 8 mM DTT. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 50% ethanol and 10% acetic acid and destained in 25% ethanol. Development of clear zones on the blue background indicated proteolytic activity. The molecular weight of a protein under investigation was estimated according to the modified method of Bryan (*24*). Sigma native molecular weight standards including thyroglobulin (M_r = 669000), amylase (M_r = 200000), and carbonic anhydrase (M_r = 29000) were separated on 5, 7.5, 10, and 12.5% acrylamide gels. The relative electrophoretic mobility (R_f) of the protein band was determined, and the logarithm of R_f was plotted against gel concentration. The slope (K_r) was determined using linear regression, and $-\log K_r$ values were plotted against log molecular weight of the standards.

Size Exclusion HPLC. Size exclusion chromatography (SEC) was performed on HPLC using a Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) connected with a Bio-Rad HPLC pump (model 2700, Bio-Rad Laboratories, Inc., Hercules, CA) and a UV detector (Bio-Rad model 1706). Fish extract, 200 μL, containing 5−10 mg of protein was injected into the column after filtration through a 0.22 μM filter to remove large particles. The proteins were eluted isocratically with 50 mM sodium phosphate buffer, pH 7.2, at a flow rate of 0.5 mL/min. Eluted proteins were monitored by absorbance at 280 nm and collected in 1.5 mL aliquots for further analyses. The caseinolytic activity of each fraction was determined at pH 5.5 and 8.0 in the presence and absence of 2.5 M urea.

The proteinase separated on SEC-HPLC was estimated for its molecular weight by plotting relative elution volume (V_e/V_0) against the logarithm of M_r of the protein standards. The elution volume (V_e) was measured for each protein standard and the proteinase, and the void volume (V_0) was estimated by the elution volume of blue dextran (M_r = 2000000). The standards used included horse heart cytochrome C (M_r = 12400), carbonic anhydrase (M_r = 29000), BSA (M_r = 66000), and sweet potato amylase (M_r = 200000).

Protein Determination. Protein concentration was determined according to the method of Lowry et al. (*17*) using BSA as a standard.

RESULTS

Autolysis in ATF Muscle. Autolytic activity of ATF muscle was characterized by temperature and pH profiles, as shown in Figures 1 and 2, respectively. The temperature profile analyzed at the natural pH of fish muscle without adjustment indicated that autolytic activity increased markedly from 37 °C to the highest peak at 60 °C before becoming rapidly inactivated at higher temperatures. The pH profile of autolytic activity analyzed at 60 °C showed a peak at pH 5.5 (Figure 2). Activity decreased rapidly with an increase in pH. However, the activity slightly increased in the pH region of 7.5−8.5 by ~60% of that observed at pH 5.5 followed by a rapid decrease at higher pH. On the basis of the temperature and pH optima, autolysis of ATF muscle was thought to be mediated by heat-activated proteinases that are optimally active at slightly acidic and alkaline pH values. The results coincide with the postmortem pH of fish muscle for flatfish, which is pH 5.5 (*25*). This may explain the severe textural degradation observed with the fish after death and the undesirable paste-like texture occurring during cooking at elevated temperatures.

Effect of Urea. The temperature profile of caseinolytic activity of fish extract was established at pH 5.5 and 8.0, respectively, at which the autolytic activity peaks were found. Optimum temperature and the magnitude of activity were affected by the addition of urea. At pH 5.5, urea shifted the optimum temperatures from 60 to 45 °C. Urea was generally found to increase the caseinolytic activity of fish extract in the temperature region below 50 °C (Figure 3). This effect was particularly evident at the assay condition of pH 8.0, on which the activity was increased throughout the temperatures studied, 0−65 °C. At the assay condition of pH 5.5, addition of 2.5 and 4.0 M urea shifted the optimum activity down to 45 °C with the slight decrease in the maximum activity at each optimum temperature (Figure 3a). At pH 8.0, urea not only shifted the optimum temperature to 55 °C but also increased the maximum activity of fish extract observed at the optimum temperature (Figure 3b). The increase in maximum activity was larger when the higher concentrations of urea were added. In the temperature range below 45 °C, where the caseinolytic activity of the control was rarely detected, the activity induction by urea was substantial, showing 20−30-fold increases by 2.5−4.0 M urea.

Effect of Proteinase Inhibitors. All four types of inhibitors, that is, serine, cysteine, aspartic acid, and metalloprotease, were tested with ATF extract to determine the class of active proteinase at pH 5.5 and 8.0.

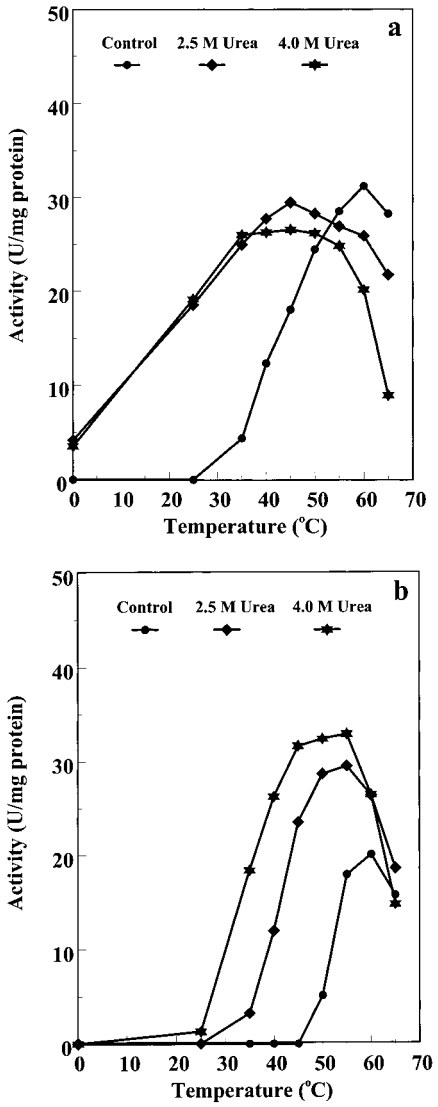

Figure 3. Effect of urea on temperature profile of caseinolytic activity of fish extract: (a) at pH 5.5; (b) at pH 8.0. Urea was added in the activity assay buffer to final concentrations of 2.5 and 4.0 M. The activity was determined by the TCA−casein method and expressed as micromoles of tyrosine released per milligram of protein.

Among the inhibitors tested, the highest inhibitions, 90−92%, at both pH conditions were shown by E-64, a specific cysteine protease inhibitor (Table 1). Leupeptin and iodoacetate, reported as both serine and cysteine protease inhibitors, also showed substantial inhibitory effects on the enzyme activity, but to a lesser extent, 74−90%. Specific inhibitors of serine proteinase (PMSF and soybean trypsin inhibitor), aspartic proteinase (pepstatin), and metalloproteinase (DTT + EDTA) showed negligible (<8.0%) inhibition of the activity. The results primarily indicate that the enzymes active at both pH values were cysteine proteinases, which is in agreement with the heat stable enzyme partially purified from ATF muscle by Wasson et al. (*16*).

Hydrolysis of Synthetic and Protein Substrates. The hydrolytic activity of the fish extract was analyzed against the synthetic substrates specific for cathepsins B, H, and L, which have been shown to be proteinases active in acidic pH ranges. To focus only on heat-activated proteinases active at pH 5.5 and 8.0, activity was measured at 55 °C. Among the substrates tested, fish extract could hydrolyze only Z-Phe-Arg-NMec, a specific substrate commonly used to assay cathepsin L activity (Table 2). The higher rate of hydrolysis at pH 5.5 clearly indicated cathepsin L is responsible for the autolytic activity of ATF muscle in the acidic pH range. At pH 8.0, Z-Phe-Arg-NMec was also found to be the only substrate being hydrolyzed by fish extract, even though the rate was lower than that of pH 5.5. On the basis of the substrate specificity and the report by Wu (*26*), we propose that the activity is due to cathepsin L complex with an endogenous proteinase inhibitor. Wu (*26*) reported that the pH optimum of P-II, a procathepsin L complex with an endogenous cysteine proteinase inhibitor, shifted from pH 4.5 to 7.0 by acidification of the enzyme at pH 3.3. Acidification is a process used to dissociate cysteine proteinase complexes from their endogenous proteinase inhibitors (*27*).

Preincubation of the enzyme extract at pH 7.5 for 1, 2, 4, and 6 h significantly decreased the hydrolytic activity of fish extract assayed at pH 5.5 against Z-Phe-Arg-NMec, but no significant effects were found when it was analyzed at pH 8.0 (Table 3). A neutral pH incubation was presumed to inactivate other lysosomal proteases with substrate specificities similar to that of cathepsin S, although it does not affect the activity of

Table 2. Hydrolytic Activity of Fish Extract on Various Synthetic Substrates

		activity[a] (units/mg of protein)	
activity	substrate used	pH 5.5	pH 8.0
cathepsin L	Z-Phe-Arg-NMec	4.30 ± 0.02	1.89 ± 0.12
cathepsin B	Z-Arg-Arg-NMec	ND	ND
cathepsin H	L-Arg-NMec	ND	ND

[a] Averages of triplicates ± SD; ND, nondetectable.

Table 3. Effect of Preincubation at 7.5 on Hydrolytic Activity of Fish Extract at pH 5.5 and 8.0 Using Z-Phe-Arg-NMec as a Substrate

	incubation time (h)	specific activity[a] (units/mg of protein)	
		pH 5.5	pH 8.0
fish extract	1	4.31 ± 0.02	1.90 ± 0.12
	2	4.18 ± 0.11	1.86 ± 0.04
	4	4.22 ± 0.14	1.82 ± 0.05
	6	4.24 ± 0.25	1.96 ± 0.02
fish extract with neutral incubation	1	4.06 ± 0.11*	1.91 ± 0.07
	2	3.86 ± 0.03*	1.82 ± 0.05
	4	3.72 ± 0.05*	1.87 ± 0.01
	6	3.48 ± 0.11*	1.82 ± 0.07

[a] Averages of triplicates ± SD. Values denoted by an asterisk are significantly different ($p < 0.05$) from those of fish extract incubated at 37 °C for the same incubation time without preincubation at pH 7.5.

Table 4. Hydrolytic Activity of Arrowtooth Flounder Fish Extract on Various Substrates

	enzymatic activity	
substrate	pH 5.5	pH 8.0
casein[a]	21.51 ± 2.37	12.78 ± 3.90
ATF myofibrils[a]	18.56 ± 1.46	11.71 ± 3.04
acid-treated hemoglobin[a]	14.44 ± 2.18	9.26 ± 3.13
BSA[a]	13.88 ± 1.54	8.93 ± 3.30
azocasein[b]	0.0310 ± 0.0009	0.0091 ± 0.0000
hide powder azure[c]	0.0200 ± 0.0000	0.0067 ± 0.0000

[a] Averages of four replicates ± SD representing nanomoles of tyrosine released per minute per milligram of protein of fish extract. [b] Average of triplicate analyses representing an increase in absorbance at 450 nm (A_{450}) per minute per 4.43 mg of protein of fish extract. [c] Average of duplicate analyses representing an increase in absorbance at 595 nm (A_{595}) per minute per 1.15 mg of protein of fish extract.

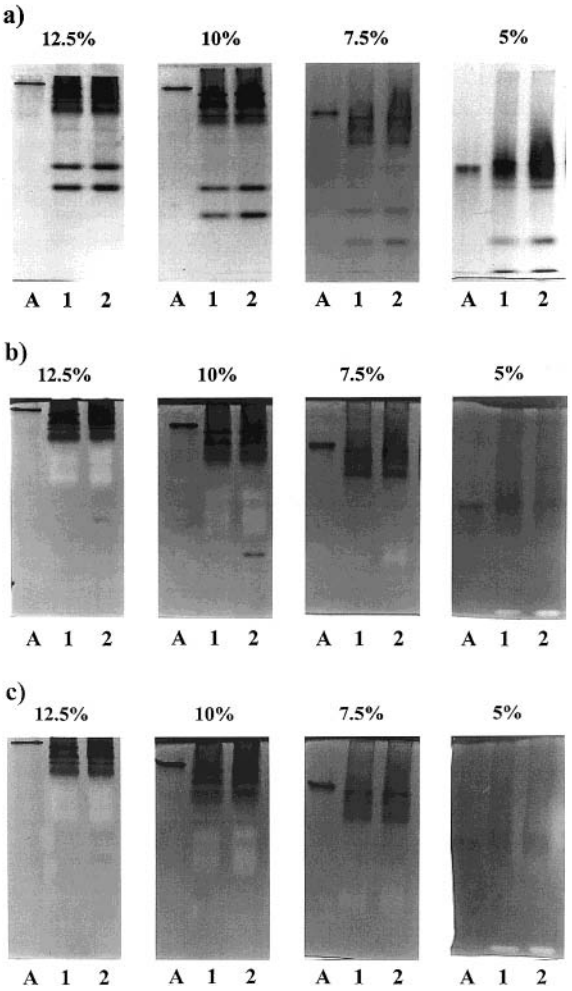

Figure 4. (a) Separation of fish muscle proteinases on native discontinuous gel electrophoresis followed by staining with Coomassie blue R250 for total proteins, as a control for activity-stained gels. Lane A represents amylase (M_r = 200000) used as a marker protein. Lanes 1 and 2 were fish extract applied at 18 and 36 μg of protein, respectively. (b) Separation of fish muscle proteinases on native discontinuous gel electrophoresis followed by staining for proteolytic activity at pH 5.5, 60 °C. Lane A represents amylase (M_r = 200000) used as a marker protein. Lanes 1 and 2 were fish extract applied at 18 and 36 μg of protein, respectively. (c) Separation of fish muscle proteinases on native discontinuous gel electrophoresis followed by staining for proteolytic activity at pH 8.0, 60 °C. Lane A represents amylase (M_r = 200000) used as a marker protein. Lanes 1 and 2 were fish extract applied at 18 and 36 μg of protein, respectively.

cathepsin S (*28*). Preincubation of the enzyme extract at 40 °C and pH 7.5 over a period of 60 min allowed determination of the activity of cathepsin S in the presence of cathepsins L and B using Z-Phe-Arg-NMec as a substrate. Thus, it can be presumed that cathepsin S may contribute to the activity detected at pH 8.0 and also autolysis at alkaline pH.

In contrast to classification of cathepsins, the alkaline protease is broadly defined on the basis of its optimal condition and its sensitivity to specific inhibitors. Due to no specific substrates being available for alkaline proteinases, hydrolytic activities of fish extract on various protein substrates were compared (Table 4). All types of protein substrates were hydrolyzed at a higher rate at pH 5.5 than at pH 8.0. For both pH conditions, casein was the most preferred substrate. Myofibrils extracted from ATF muscle showed a hydrolysis rate comparable to that of casein, 86.3 and 91.6% at pH 5.5 and 8.0, respectively. The hydrolysis rates of acid-treated hemoglobin and BSA were only 65−73% that of casein. Hydrolytic activity at pH 5.5 against azocasein was 3.4-fold that at pH 8.0. The low hydrolysis rate observed at pH 8.0 compared to those of other substrates implied that azocasein may not be a suitable substrate for determining the activity of the proteinase active in alkaline pH values. Collagenolytic activity determined using hide powder covalently linked with Remazolbrilliant Blue (*21*) showed that the fish extract was more active at pH 5.5 than at pH 8.0.

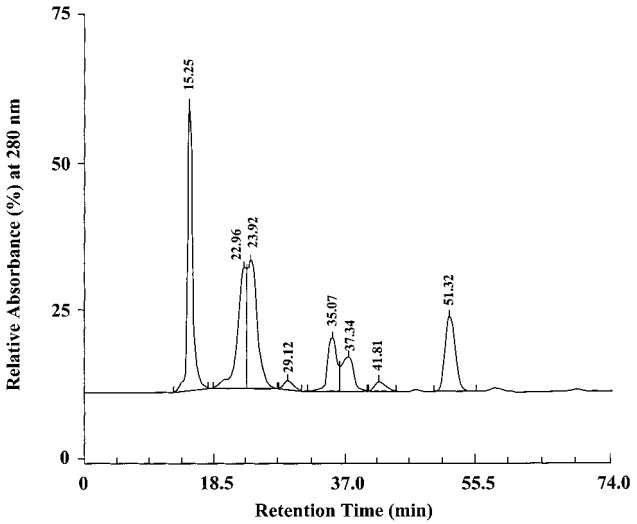

Figure 5. Elution profile of ATF crude extract on Superose 12 HR 10/30. Fish proteins, 250 μg, were applied and eluted with 50 mM sodium phosphate buffer, pH 7.2, at a flow rate of 0.5 mL/min. The eluted proteins were monitored spectrophotometrically by absorbance at 280 nm.

Activity-Stained Substrate Gel Electrophoresis. The proteinase activity in fish extract was identified by separation on native discontinuous substrate polyacrylamide gels followed by staining for protein (Figure 4a) and proteolytic activity (Figure 4b,c). Activity-stained substrate gels of muscle extract incubated at pH 5.5 and 8.0 revealed similar patterns of activity bands shown as clear zones on the dark background (Figure 4b,c). The molecular weights of the bands were estimated to be in the range of 43−124 kDa. The obtained results indicated that fish extract contained several proteinases active at pH 5.5 and 8.0, and the proteinases being involved in both conditions may be identical on the basis of the activity-stained gels (Figure 4b,c).

Separation of Fish Extract on SEC-HPLC. SEC-HPLC was employed as a method of choice, as it can separate proteins on the basis of their size on non-denaturing condition and thus is capable of differentiating a high molecular weight alkaline protease from lower molecular weight cathepsins. When ATF extract was separated on SEC-HPLC (Figure 5), only one proteolytic peak was observed. This peak, eluted at a retention time of 26 min, revealed the previously observed effects of urea, reduction of activity by the addition of urea at pH 5.5 (Figure 6a), and enhancement at pH 8.0 (Figure 6b). Its molecular weight was estimated to be M_r = 39800 on Superose 12 HR 10/30 (Figure 7).

DISCUSSION

Autolysis of ATF muscle at elevated temperatures is due to a heat-activated proteinase active at both acidic

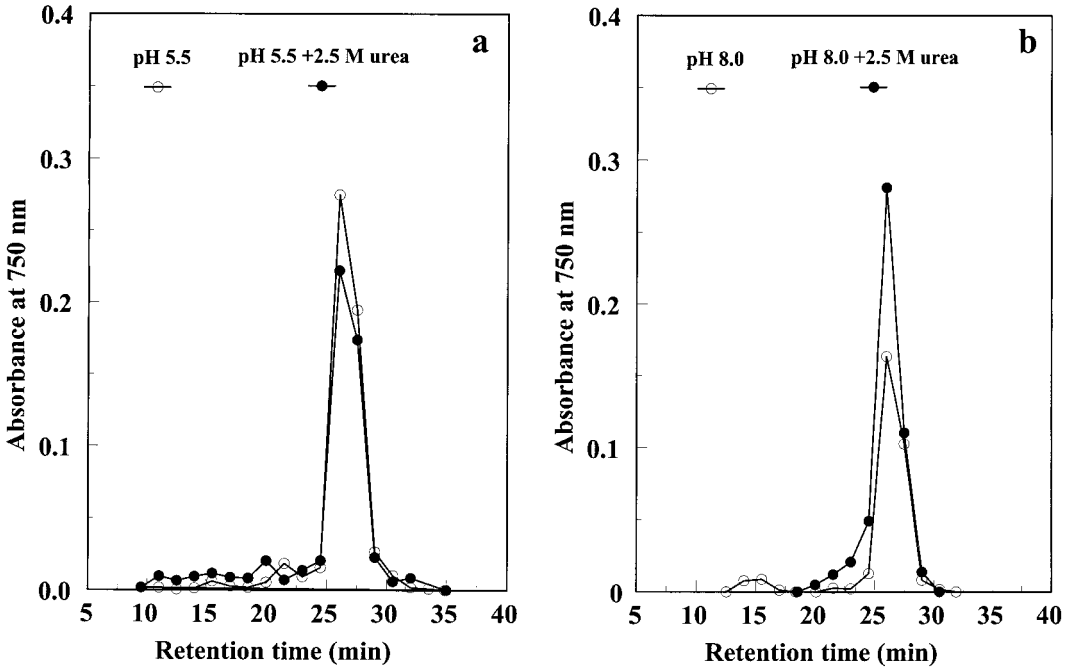

Figure 6. Caseinolytic activity of Superose 12 HR 10/30 fractions of ATF crude extract: (a) assayed at pH 5.5 and 60 °C in the presence and absence of 2.5 M urea; (b) assayed at pH 8.0 and 60 °C in the presence and absence of 2.5 M urea. Data are shown only for elution time <40 min, when proteolytic activity was detected.

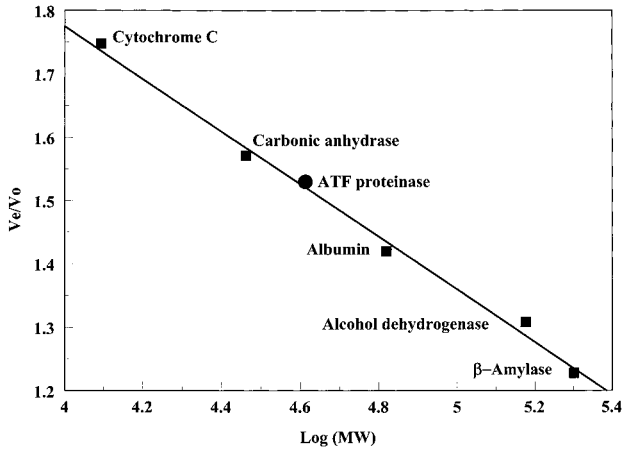

Figure 7. Estimation of the native molecular weight of ATF proteinase based on elution volumn on Superose 12 HR 10/30 column. The molecular weight was estimated to be $M_r = 39800$.

and alkaline pH values. The obtained results clearly indicated that cathepsin L is the active proteinase in ATF extract and is primarily responsible for the autolysis of fish muscle at slightly acidic pH. Cathepsin L has been reported as a major proteinase in chum salmon (*9*) and Pacific whiting muscle (*18*). Cathepsin L degrades proteins at least 10 times more rapidly than the other cysteine proteinases, including cathepsins B and H (*29*) and is very active in degrading myofibrillar proteins. The fact that the molecular activity of cathepsin L with myosin is 10 times greater than that of cathepsin B (*30*) may explain the complete myosin degradation during autolysis of ATF muscle at 60 °C. In Pacific whiting, high protease activity and textural degradation are associated with the infection of Myxosporean parasites (*31*). However, no clear relationship between parasite density and textural deterioration of the cooked fillets was observed in ATF (*2*). Cathepsin L was shown to be capable of hydrolyzing various types of protein substrates. In addition to myofibrillar proteins, cathepsin L was reported to have very high activity against various collagens and elastin (*32*). Thus, it is presumed to cause partial disintegration of the original extracellular matrix structure, which may play an important role in tissue softening of fish.

Among the cathepsin L-like enzymes, the presence of cathepsin S was presumed for the remaining activity of fish extract after preincubation at pH 7.5. Cathepsin S is the only member of the lysosomal cathepsins that can retain proteolytic activity after prolonged exposure to neutral pH (*33*, *34*). Cathepsin S is a lysosomally located, single-chain, cysteine proteinase with a high endopeptidase activity against proteins including elastin and collagen (*35*). Although its substrate specificity shows some similarities with cathepsin L, it was shown by biochemical and immunological methods and analysis of the amino acid sequences that cathepsin S and cathepsin L are distinct enzymes (*36*). Therefore, the autolytic activity or proteolytic activity on various substrates detected at pH 8.0 is apparently due to Z-Phe-Arg-NMec hydrolyzing activity mediated by cathepsin S.

Depending upon pH, the distinctive response to urea may explain the presence of heat stable alkaline protease in fish extract, even though its contribution may be less than that of cathepsins. Because it exhibits little or no catalytic activity unless assayed at a nonphysiological high temperature or activated by protein-denaturing agents such as urea, the enzyme will contribute to the post-mortem degradation of muscle only when denaturing substances analogous to urea are produced during storage. The activation phenomenon was previously reported by Toyohara et al. (*15*) and Kinoshita et al. (*37*) as a unique characteristic of alkaline proteases that are known to be predominantly active at the physiological condition of marine flesh tissues. However, several studies have shown that cathepsins L and S are stable or activated in the presence of urea (*28*). Proteinase activity of cathepsin L against azocasein was increased in the presence of 3

M urea and was used as a unique characteristic to differentiate cathepsins L and B (*38*). Recently, the activity of cathepsin L recovered from Pacific whiting surimi wash water against Z-Phe-Arg-NMec was found to be highly enhanced by the addition of 2.5 M urea (*39*). Thus, cathepsin L, cathepsin L-like enzyme, or cathepsin L complex might exhibit hydrolytic activity on various protein substrates at both pH 5.5 and 8.0 and showed identical responses to urea, which were often used to characterize alkaline protease.

Identical activity bands resolved on the activity-stained substrate gels at both pH 5.5 and 8.0 imply the involvement of several proteinases in the proteolytic activity. However, only one activity peak was obtained on SEC-HPLC. This fraction was thought to be the most predominant proteinase found in ATF extract. This fraction also showed the previously shown effects of urea: reduction of activity at pH 5.5 by the addition of urea but enhancement at pH 8.0. On the basis of its estimated molecular weight of $M_r = 43000$ by activity-stained substrate gel electrophoresis or $M_r = 39800$ by SEC-HPLC, it is most likely to be cathepsin L. Cathepsins L and S are relatively small when compared to the alkaline proteinase. The molecular weights of purified cathepsins L and S are estimated to be 29 and 24 kDa, respectively (*32*). However, they are often found to be complexed with endogenous inhibitors, such as cystatins and α-cysteine proteinase, which apparently increase the molecular weight of the proteinases by 11−14 kDa (*27*, *40*, *41*). Cathepsin L has been found in a complex form with its natural inhibitor; therefore, dissociation of the cathepsin L−inhibitor complex upon addition of urea may not be ruled out.

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