Purification and Characterization of Pacific Whiting Proteases

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Two forms (P-I and P-II) of a protease were purified to 560- and 21.5-fold from the sarcoplasmic fluid of Pacific whiting (*Merluccius productus*). The enzymes were assayed by hydrolysis of azocasein at 55 °C and pH 5.5. Both forms were composed of a single polypeptide of M_r 28 800; however, P-I also contained two low M_r components which were removed by low pH treatment prior to size exclusion chromatography. P-I and P-II showed pH optima at 5.5 and 6.0 and temperature optima of 55 and 60 °C, respectively. The enzymes had an identical isoelectric point of 4.91 with a minor band at 4.94. The proteases had high activity toward Z-Phe-Arg-NMec and casein. The activity of both forms was inhibited by all sulfhydryl reagents tested as well as the cathepsin L-specific inhibitor Z-Phe-Phe CHN_2 and activated by reducing agents, suggesting the enzymes are forms of cathepsin L.

Keywords: *Protease; proteolytic; cathepsin; surimi*

INTRODUCTION

Pacific whiting (*Merluccius productus*) is the most abundant fishery resource off the Northwest coast of the contiguous United States, with annual harvests ranging from 150 000 to 250 000 metric tons (Radtke, 1992). Due to the soft texture associated with proteolysis, diverse utilization of the species has been impeded. At present, the majority of harvested Pacific whiting is used for the production of surimi. Surimi, the raw ingredient in seafood analogs, is prepared from minced and washed fish flesh. Food-grade protease inhibitors have been found to limit the amount of proteolytic degradation of the muscle proteins (Morrissey et al., 1993). This improves the gel strength of surimi and increases its market potential.

Pacific whiting have been known to suffer from infection by the Myxosporean parasite, *Kudoa paniformis* (Adlerstein and Francis, 1991). Studies have shown correlations between the degree of infection and proteolytic activity (Konagaya and Aoki, 1981; Kudo et al., 1987; Patashnik et al., 1982). It was hypothesized that the protease responsible for tissue breakdown of Pacific whiting was of parasitic origin (Konagaya and Aoki, 1981). However, this hypothesis has been challenged due to the absence of enzyme-producing organelles in spores (Stehr and Whitaker, 1986).

Cathepsins are mostly present in the lysosomes of phagocytes and are involved in cellular immunity induced by pathological conditions. Post-mortem release of cathepsins from lysosomes results in hydrolysis of muscle proteins (Asghar and Bhatti, 1987; Etherington, 1984). Cathepsin D has been shown to be involved in the post-mortem proteolysis occurring in tilapia muscle (Jiang et al., 1990). Cathepsins B and C have been implicated in the proteolysis of infected fish flesh of Pacific whiting as shown by protease inhibitor studies and pH optima of these enzymes (Erickson et al., 1983). A cathepsin L-like protease purified from Pacific whiting was shown to be responsible for breakdown of the muscle protein (Masaki et al., 1993). Cathepsins were also reported to be responsible for tissue softening of many other fish species, including croaker and arrowtooth flounder (Greene and Babbitt, 1990; Su et al., 1981). Martone et al. (1991) isolated a trypsin-like protease from Peruvian hake (*Merluccius hubbsi*) which was optimally active at pH 7–9 on azocasein at 37 °C. The enzyme degraded major contractile and cytoskeletal constituent proteins of myofibrils at pH 7. More recently, Wasson et al. (1992) characterized a protease in the muscle of arrowtooth flounder at 55 °C. The enzyme was optimally active at pH 6–7 toward casein and was inhibited by sulfhydryl reagents. Hara et al. (1988) also reported the presence of cysteine protease activity in carp muscle. Most of the protease reported, however, appeared to be incapable of affecting the type of textural degradation observed in Pacific whiting induced by cooking.

Previously, Morrissey et al. (1993) showed that myosin was the major protein in Pacific whiting surimi hydrolyzed by protease during cooking at 60 °C. We found that proteolytic activity of Pacific whiting muscle on azocasein was maximal at pH 5.5 and 55 °C (An et al., 1994a). The objective of this study was to purify the protease responsible for tissue softening and weakening of surimi gel strength of Pacific whiting.

MATERIALS AND METHODS

Materials. Pacific whiting were caught off the northern Oregon coast in the summer of 1992, packed in ice, and transferred to the Oregon State University Seafood Laboratory within 24 h of capture. Fish were filleted, vacuum-packed, and frozen at −20 °C until used. *N*-Carbobenzoxy-Phe-Arg 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), Z-Arg-Arg-NMec, L-Arg-NMec, leupeptin, pepstatin A, soybean trypsin inhibitor, azocasein, and 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane (E-64) were purchased from Sigma Chemical Co. (St. Louis, MO). Carbobenzoxy-Phe-Phe-diazomethyl ketone (Z-Phe-Phe CHN_2) was purchased from Enzyme Systems Products (Dublin, CA). α_2-Macroglobulin and cystatin were purchased from Calbiochem (La Jolla, CA). Sodium caseinate was purchased from US Biochemical Corp. (Cleveland, OH). Butyl-Sepharose was purchased from Pharmacia (Piscataway, NJ). DEAE Bio-Gel A was purchased from Bio-Rad Laboratories (Richmond, CA).

Purification of Pacific Whiting Protease. Parasitized Pacific whiting fillets had higher proteolytic activity than nonparasitized fish and were used as starting material for the purification. The fillets were finely comminuted and centri-

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fuged at 5000g for 30 min to obtain sarcoplasmic fluid. The supernatant was then diluted with an equal volume of McIlvaine's buffer (0.2 M sodium phosphate−0.1 M citric acid) at pH 5.5 and heated at 60 °C for 3 min. After centrifugation at 7800g for 15 min, the supernatant was collected and dialyzed in 20 mM Tris, pH 7.5, starting buffer (SB). Prior to application of the sample to a butyl-Sepharose column (2.6 × 20 cm) at room temperature, saturated ammonium sulfate (AS) was added to adjust the concentration to 1 M. After the column was washed with 1 M AS in SB until A_{280} was less than 0.05, fractions (5 mL) with protease activity were eluted with SB at a flow rate of 0.5 mL/min. Fractions were assayed for activity at pH 5.5 and for protein content. The fractions were pooled on the basis of activity into two separate pools, designated P-I and P-II. Fraction pools were dialyzed against 20 mM Tris, pH 7.5, at 4 °C for DEAE Bio-Gel A chromatography. Samples were applied at 4 °C to a DEAE Bio-Gel A column (2.6 × 10 cm) previously equilibrated with SB. After washing overnight with SB, active fractions were eluted with a linear gradient (500 mL) of 0−300 mM NaCl. The fractions were pooled and assayed for activity at pH 5.5 and protein content. The fraction pool was then concentrated by ultrafiltration using a Centriprep 10 cartridge (Amicon, W. R. Grace & Co.-Conn., Beverly, MA) at 4 °C. Concentrated fractions of P-I were subjected to low-pH treatment. Both low pH treated and untreated samples were further purified by SEC-HPLC, as described in a later section.

Enzyme Assays. Protease activity was assayed during the purification by the TCA−azo method (An et al., 1994a) with McIlvaine's buffer, pH 5.5, containing 2 mM β-mercaptoethanol (βME) with azocasein as a substrate. All activity assays were carried out at 55 °C, unless otherwise indicated. Blank was prepared in the same manner except that enzyme was added right before TCA precipitation, and activity was determined as the increase in A_{428} compared with that of blank (ΔA_{428}).

Activity on casein was determined as tyrosine (Tyr) equivalents solubilized in TCA supernatants as measured by the Lowry assay (Lowry et al., 1951). One unit of activity was defined as that releasing 1 nmol of Tyr/min.

Specific activity of purified enzyme was determined on the synthetic substrates, Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and L-Arg-NMec, according to the method of Barrett and Kirschke (1981). A unit of activity on the fluorogenic substrates was expressed as 1 nmol of methylcoumarin released/min. Fluorescence was determined by excitation at 370 nm and emission at 460 nm on an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD). The molar concentration of active enzyme was measured by titration with E-64 according to the method of Barrett and Kirschke (1981).

Protein Determination. Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. Absorbance at 280 nm was used to follow protein concentration during the purification.

Characterization of Pacific Whiting Protease. *SDS−PAGE.* Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). SEC-HPLC fraction pool of P-I (18 μg), SEC-HPLC fraction of P-I subjected to low-pH treatment (10 μg), and Centriprep 10 concentrated DEAE fraction of P-II (10 μg) were applied and separated on a 15% SDS−PAGE gel and stained with 0.125% Coomassie brilliant blue R-250. The molecular weight of the purified enzyme(s) was determined using a low molecular weight calibration kit (Pharmacia-LKB Biotechnology, Piscataway, NJ), to which aprotinin (Sigma) was added to allow estimation of low M_r components.

Isoelectric Focusing. The isoelectric point was determined by isoelectric focusing in premade agarose gels, pH range 3−10 (FMC Corp., Rockland, ME) using a broad range pI standard (Pharmacia). Bands were visualized by staining with 0.125% Coomassie brilliant blue R-250 as recommended by Bio-Rad. The isoelectric point was calculated by linear regression.

Size Exclusion Chromatography. Size exclusion chromatography was carried out on a Superose 12 (Pharmacia LKB) fitted to a Bio-Rad HPLC pump (Model 2700) with a UV detector (Bio-Rad Model 1706). Samples (500 μL) were eluted at a flow rate of 0.8 mL/min in SB with 0.1 M NaCl. Absorbance at 280 nm was monitored. Fractions of 0.5 mL were collected for determination of protease activity and protein content.

Low pH Treatment. To promote dissociation of enzyme−inhibitor complexes, equal volumes of McIlvaine's buffer, pH 3.5, were added to concentrated DEAE Bio-Gel A fractions. Fractions were then incubated for 10 min at 25 °C prior to SEC-HPLC separation.

Optimal pH and Temperature. Purified proteases were assayed in McIlvaine's buffer in the pH range 3.5−8.0, with 2 mM βME at 55 °C for 15 min. The temperature optima were determined by assaying purified proteases for 15 min at various temperatures between 22 and 70 °C in McIlvaine's buffer at pH 5.5 for P-I or at pH 6.0 for P-II.

Inhibitor Study. The purified protease was incubated with various inhibitors in McIlvaine's buffer, pH 6.0, with 2 mM βME for 10 min at room temperature prior to activity assay with azocasein. The residual activity was expressed as a percentage of the enzyme activity with added inhibitor compared to the control.

Table 1. Purification Table of Pacific Whiting Protease

step	total protein (mg)	total act. (U)[a]	sp act. (mU/mg)	yield (%)	fold purity
sarcoplasmic fluid	4980	1380	277	100	1.0
heat treatment	1060	1210	1140	87.0	4.1
butyl-Sepharose					
P-I	74.8	335	4480	24.0	16.2
P-II	64.5	365	5660	26.0	20.4
DEAE Bio-Gel A					
P-I	38.2	264	6910	19.0	24.9
P-II	39.0	188	4820	14.0	17.4
Centripep 10					
P-I	18.6	422	22700	31.0	81.9
P-II	7.5	45	5960	3.2	21.5
SEC-HPLC, P-I					
acidified			158000[b]		570[b]
not acidified			37100[b]		134[b]

[a] One unit of activity was defined as 1 nmol of Tyr equivalent solubilized/min. [b] Estimated by active-site titration.

RESULTS

Purification of Pacific Whiting Protease. Purification steps of protease are summarized in Table 1. Heat treatment of sarcoplasmic fluid was used to exploit the thermostability of the enzyme and to denature and remove heat-labile myofibrillar and sarcoplasmic proteins. This treatment also served to inactivate other heat-labile proteolytic enzymes.

Hydrophobic chromatography on butyl-Sepharose served to separate activity into two peaks designated P-I and P-II (Figure 1), which were then purified separately. Mason et al. (1984) reported that hydrophobic chromatography on phenyl-Sepharose was highly effective in concentrating cathepsin L activity. Difficulty in eluting these enzymes after adsorption onto phenyl-Sepharose required us to use the less hydrophobic butyl-Sepharose in this study. P-I eluted very early by 20 mM Tris, pH 7.5, along with the major protein peak. P-II eluted later than P-I in the absence of a significant amount of contaminating protein as indicated by A_{280} (Figure 1).

On DEAE Bio-Gel A (Figures 2 and 3), a 1.5-fold purification for P-I was achieved. An increase in total activity was observed during concentration of DEAE fractions of P-I by ultrafiltration with Centriprep 10 (M_r

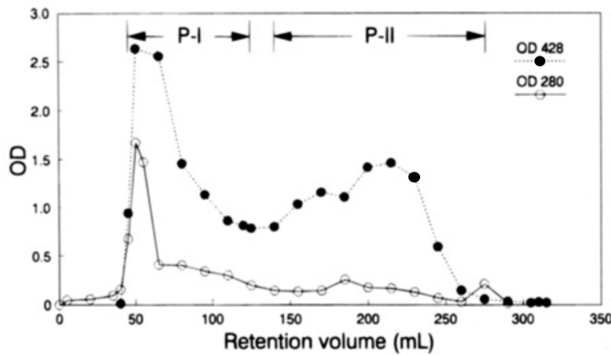

Figure 1. Butyl-Sepharose column elution profile of heat-treated Pacific whiting sarcoplasmic fluid. Enzyme solution was adjusted to 1 M AS in SB and applied to a butyl-Sepharose column (2.6 × 20 cm) at room temperature. After the column was washed with 1 M AS in SB, elution was carried out with SB. Fractions of 5 mL were collected at a flow rate of 0.5 mL/min. Activity as determined by TCA–azo assay is expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

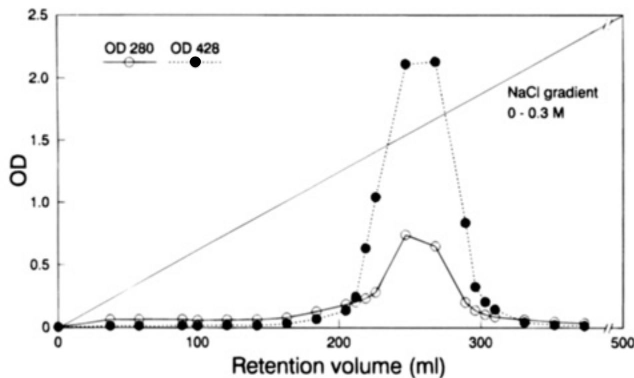

Figure 2. Elution profile of P-I on DEAE Bio-Gel A. Pooled fractions from butyl-Sepharose chromatography were dialyzed into SB and applied onto a DEAE Bio-Gel A column (2.6 × 10 cm) at 4 °C. Fractions (7 mL) were eluted with a linear NaCl gradient from 0 to 300 mM. Activity was determined by TCA–azo assay and expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

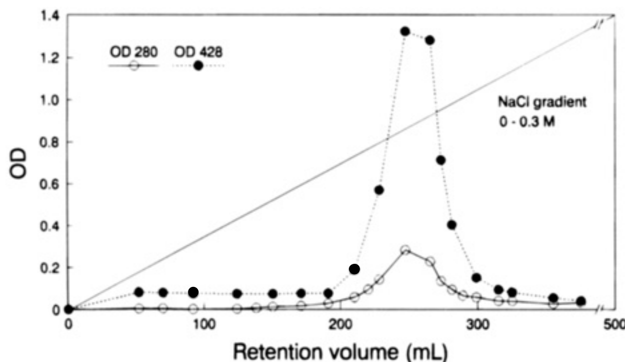

Figure 3. Elution profile of P-II on DEAE Bio-Gel A. Pooled fractions from butyl-Sepharose chromatography were dialyzed into SB and applied onto a DEAE Bio-Gel A column (2.6 × 10 cm) at 4 °C. Fractions (4.5 mL) were eluted with a linear NaCl gradient from 0 to 300 mM. Activity was determined by TCA–azo assay and expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

10 000 cutoff), which could be explained by loss of an inhibitor of low M_r. The purification data for P-II seem to indicate that DEAE chromatography did not accomplish any further purification. However, P-II was not stable during purification, resulting in the low purification fold and yield obtained.

Molecular Properties. By SEC-HPLC, the chromatogram of P-I consisted of an overlapped double peak

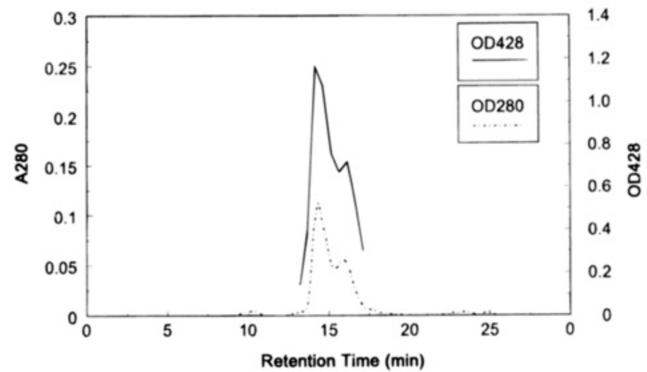

Figure 4. Superose 12 HPLC column elution profile of P-I. Samples were eluted at a flow rate of 0.8 mL/min in 20 mM Tris, pH 7.5. Activity was determined by TCA–azo assay and expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

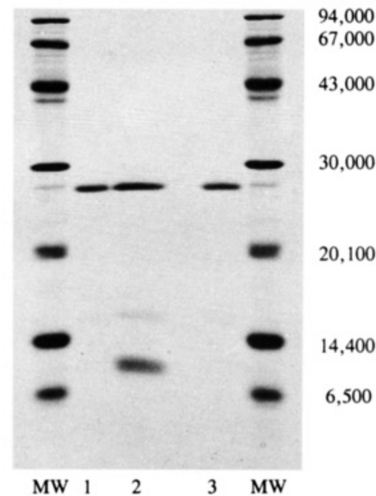

Figure 5. SDS–PAGE (15% T) of purified Pacific whiting proteases. Proteins were visualized by staining with 0.125% Coomassie brilliant blue R-250. Lane 1, SEC-HPLC fraction of P-I (acidified prior to HPLC), 10 μg; lane 2, SEC-HPLC fraction of P-I, 18 μg; lane 3, DEAE fraction of P-II, 10 μg; MW, molecular weight protein standards.

of protein with retention times of 14.28 and 15.86 min (Figure 4). Fractions from both peaks contained active enzyme as determined by TCA–azo assay (Figure 4) and were pooled for analysis by SDS–PAGE. These pooled fractions were shown to contain polypeptides of M_r 28 800, 14 500, and 9700 (Figure 5). When the pH of the sample was reduced to 3.5 prior to application onto the HPLC size exclusion column, a single protein peak containing active enzyme eluted with a retention time of 15.96 min. Analysis of fractions of this peak by SDS–PAGE revealed a single band of M_r 28 800 (Figure 5). The low-pH treatment apparently served to dissociate the low M_r components. An increase in activity of approximately 50% was observed after low-pH treatment, suggesting that dissociation of a peptide inhibitor(s) had occurred. Reassociation apparently did not occur under the conditions of SEC-HPLC, thus allowing subsequent removal of these components. These steps achieved a 6.9-fold purification when active enzyme concentration was determined by active site titration (Table 1). Previously, low-pH treatment was shown to be an effective method to dissociate an inhibitor from cathepsin L (Mason et al., 1985; Yamashita and Konagaya, 1992). The activity of the purified enzyme subsequently decayed rapidly at 4 °C. Cathepsin L was previously reported to be unstable at pH above 7.0

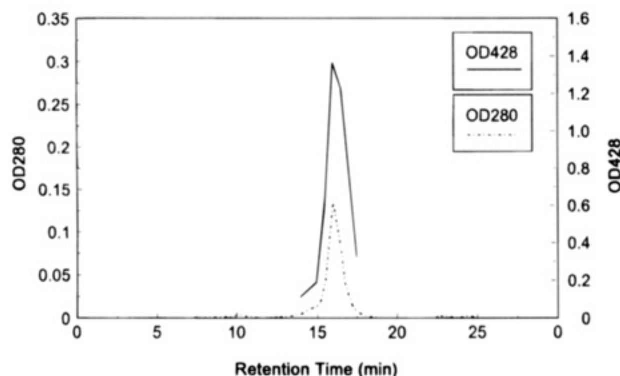

Figure 6. Superose 12 HPLC column elution profile of P-II. Samples were eluted at a flow rate of 0.8 mL/min in 20 mM Tris, pH 7.5. Activity was determined by TCA–azo assay and expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

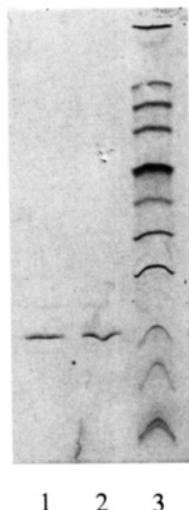

Figure 7. Isoelectric focusing of purified P-I and P-II. Lane 1, purified P-I; lane 2, purified P-II; lane 3, broad range p*I* standards (from top: trypsinogen, p*I* 9.3; lentil lectins, p*I* 8.65, 8.45, 8.15; myoglobin-basic, p*I* 7.35; myoglobin-acidic, p*I* 6.85; carbonic anhydrase B (human), p*I* 6.55; carbonic anhydrase B (bovine), p*I* 5.85; β-lactoglobulin A, p*I* 5.2; trypsin inhibitor, p*I* 4.55; amylglucosidase, p*I* 3.5).

(Mason et al., 1985). Relative retention times of the enzyme on the Superose 12 size exclusion column were dependent on ionic strength; as such, no reliable estimate of relative molecular mass (M_r) could be obtained.

The SEC-HPLC chromatogram of P-II showed one peak at 16.01 min (Figure 6). Acid treatment did not increase the activity of P-II, nor was any further purification of P-II achieved by low-pH treatment followed by SEC-HPLC. The P-II DEAE fractions did not contain any low MW components and were composed of a single polypeptide of M_r 28 800 as shown by SDS–PAGE analysis (Figure 5).

Isoelectric focusing patterns of the purified enzymes were identical, showing a major band at p*I* 4.91 and a minor band at 4.94 (Figure 7). Kirschke et al. (1977) reported multiple forms of cathepsin L from rat liver lysosomes in the pH range 5.8–6.1. Mason et al. (1984) also reported that rabbit liver cathepsin L had three main isoenzymic forms with p*I* values ranging from 5.0 to 5.9 with a whole range of minor isoenzymic forms with p*I* values ranging from 5.0 to 5.9. The authors speculated that these multi-isoenzymic forms might exist *in vivo*, and some of these forms might be due to limited proteolysis.

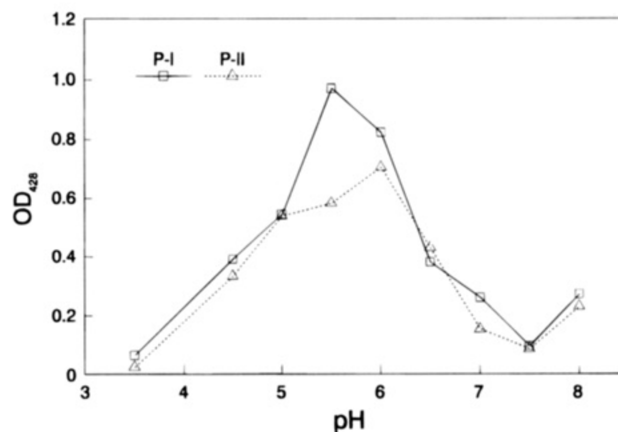

Figure 8. pH optima of purified P-I and P-II. Purified protease was assayed in McIlvaine's buffer in the pH range 3.5–8.0 with 2 mM βME at 55 °C for 15 min. Activity determinations by TCA–azo assay were carried out in duplicate at each pH.

Properties of Pacific Whiting Protease. *pH and Temperature Optima.* The pH and temperature optima of the purified protease were evaluated by azocasein at 55 °C. The enzyme exhibited an optimum at pH 5.5 and 6.0 for P-I and P-II, respectively (Figure 8). The optimal pH values of these proteases were similar to that of cathepsin L obtained from salmon, which had an optimum of pH 5.6 on Z-Phe-Arg-NMec (Yamashita and Konagaya, 1990a), and to those of cathepsin L and cathepsin L-like proteases obtained from mackerel, with optima of pH 5.0 and 5.5 on Z-Phe-Arg-NMec, respectively (Lee et al., 1993). Optimal pH for activity on protein substrates shows more variation. Okitani et al. (1980) reported that cathepsin L from rabbit muscle had a maximal activity at pH 7.0 in removing the Ca^{2+} sensitivity of myofibrils. Cathepsin L from rabbit liver had pH optima on azocasein and collagen of pH 5.2 and 3.3, respectively (Mason et al., 1984). Differences in optimal pH have been attributed to the accessibility of the substrate to the active site by charge on the substrate as well as on the active site at that particular pH environment (Mason et al., 1984). The sharp decrease in hydrolysis of azocasein by Pacific whiting protease at low pH, however, may be attributed to the decrease in solubility of the substrate below pH 4.5 as shown by An et al. (1994a).

Temperature had a marked influence on the activity with a maximum at 55 and 60 °C for P-I and P-II, respectively (Figure 9). The enzyme activity at the optimum was 5-fold greater than that at room temperature. The degradation of myosin in Pacific whiting surimi has been shown to occur most rapidly at 55 °C (Chang-Lee et al., 1989). This indicates that this enzyme activity is probably responsible for weakening the surimi gel strength by degrading the major gel-forming protein, myosin, at that temperature. The Pacific whiting proteases have slightly higher optimal temperatures compared to previously described cathepsins. Cathepsin L and cathepsin L-like protease of mackerel had optimal temperatures of 50 and 40 °C, respectively (Lee et al., 1993). Cathepsins B, H, and L of rat liver had temperature optima of 50 °C with azocasein (Kirschke et al., 1976). Cathepsins B and D of surf clams had temperature optima of 45 °C (Chen and Zall, 1986).

Effects of Activators and Inhibitors. The effects of the various activators and inhibitors on protease activity were examined (Table 2). Enzyme activity was en-

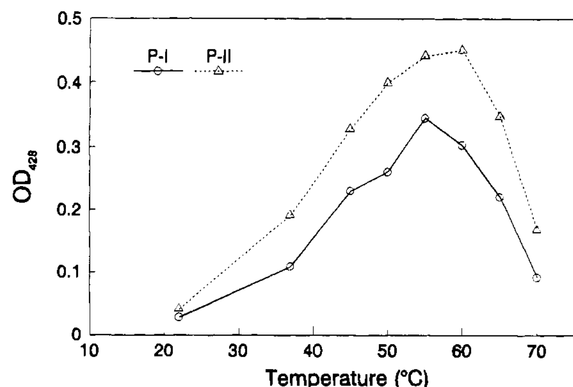

Figure 9. Temperature optima of purified P-I and P-II. The temperature optimum was determined by assaying purified protease for 15 min at various temperatures between 22 and 70 °C in McIlvaine's buffer at pH 5.5 for P-I or at pH 6.0 for P-II. Activity determinations by TCA–azo assay were carried out in duplicate at each temperature.

Table 2. Effects of Various Activators and Inhibitors on the Activity of Pacific Whiting Protease (P-I)

compound	concn (mM)	rel act. (%)
control		100
$HgCl_2$	0.01	6
p-CMB	0.01	2
$CaCl_2$	5	92
EDTA	1	116
DTT	1	248
DTT/EDTA	1	281
E-64	0.01	1
soybean trypsin inhibitor	0.1	131
leupeptin	0.01	0
pepstatin A	0.01	105
α_2-macroglobulin	0.01	68
cystatin	0.5	27
Z-Phe-Phe CHN_2	0.1	8, 4[a]

[a] P-II.

hanced by the thiol-activating agents dithiothreitol (DTT) and EDTA. EDTA was used for specific activation of cysteine proteases. The enzyme activity was inhibited by the thiol-blocking agents $HgCl_2$, *p*-(chloromercuri)benzoate, E-64, and leupeptin. Partial inhibition was observed for the polypeptide inhibitors bovine α_2-macroglobulin and chicken egg white cystatin, respectively. All of these results are consistent with the enzyme being a thiol protease. Inhibition of both enzyme forms was also observed at 0.5 mM with the active site directed protease inhibitor Z-Phe-Phe CHN_2, which has a 2000-fold higher affinity for cathepsin L than for cathepsin B from rat and human (Kirschke and Shaw, 1981). Activity of cathepsin H is (2×10^6)-fold less affected than that of cathepsin L by this inhibitor.

Substrate Specificity. To further identify the cysteine protease, we tested the activity of the purified proteases on specific substrates for cathepsins B, H, and L (Table 3), which are the most active cysteine lysosomal enzymes (Barrett and Kirschke, 1981). Both P-I and P-II were highly active on Z-Phe-Arg-NMec, which is considered to be the most specific synthetic substrate for cathepsin L. Cathepsin B, however, also can degrade this substrate to a lesser degree. Specific activities of rat liver cathepsins L and B on Z-Phe-Arg-NMec were reported to be 1229 and 400 U/μmol enzyme, respectively, while those on Z-Arg-Arg-NMec were 2.4 and 367 U/μmol, respectively (U defined as 1 μmol of peptide hydrolyzed/min at 40 °C; Kirschke et al., 1984). Therefore, the measurement of contaminating cathepsin B activity can be made on Z-Arg-Arg-NMec, since

Table 3. Substrate Specificity of Pacific Whiting Protease at 55 °C

substrate	act. (U/nmol)[a] P-I	act. (U/nmol)[a] P-II
Z-Phe-Arg-NMec	453	526
Z-Arg-Arg-NMec	0.1	0.2
L-Arg-NMec	0	0.1
casein	1050	1283

[a] One unit of activity was expressed as 1 nmol of methylcoumarin released/min on synthetic substrates or 1 nmol of Tyr/min on casein.

cathepsin L activity is negligible on that substrate. Both enzymes had less than 1 U/nmol activity on Z-Arg-Arg-NMec, indicating no contamination of cathepsin B in the purified proteins. Similarly, neither enzyme was active on the cathepsin H-specific substrate, L-Arg-NMec. Both enzymes were able to hydrolyze casein readily (Table 3). The purified enzymes were also highly active on azocasein, as this was the substrate used to follow the activity during the purification. Cathepsin L is reported to be highly active on azocasein with a 13 times higher specific activity than cathepsin B_1 from rat liver (Barrett and Kirschke, 1981; Kirschke et al., 1977).

DISCUSSION

A number of efforts have been made to characterize the protease causing soft texture of Pacific whiting. Konagaya and Aoki (1981) reported the presence of a thiol protease that was neither cathepsin B nor D. Erickson et al. (1983) compared an array of enzymic activities in Pacific whiting to that of a firm-textured fish, true cod, and suggested that cathepsin B or C may be involved. Masaki et al. (1993) purified protease from Pacific whiting and characterized the activity as cathepsin L-like.

Our purification method was designed to purify the most active endopeptidase at elevated temperatures, on the basis of previous reports that Pacific whiting undergoes significant textural breakdown during prolonged baking (Patashnik et al., 1982) and loss of surimi gel strength around 55 °C (Chang-Lee et al., 1989; Morrissey et al., 1993). Assay conditions were chosen on the basis of our previous results that maximum hydrolytic activity in the sarcoplasmic fluid of Pacific whiting occurred on azocasein at pH 5.5 and 55 °C (An et al., 1994a). In this study, the optimal pH values of the purified enzymes on azocasein were found to be 5.5 and 6.0 and the optimal temperatures 55 and 60 °C for P-I and P-II, respectively. These properties are similar to those of proteolytic activity in Pacific whiting muscle characterized by the previous observations.

It has been established that both enzymes purified in this study are forms of cathepsin L, on the basis of the activity patterns with various substrates and the effect of various chemical inhibitors. The presence of a thiol group in the active site was established by the use of specific thiol-blocking reagents. Treatment with the cathepsin L-specific inhibitor, Z-Phe-Phe CHN_2, resulted in almost complete inactivation. The enzymes had similar specific activities on Z-Phe-Arg-NMec and Z-Arg-Arg-NMec to cathepsin L of salmon (Yamashita and Konagaya, 1990a) but differed from cathepsin B with respect to activity on Z-Arg-Arg-NMec. The lack of aminopeptidase activity was demonstrated by an inability to hydrolyze L-Arg-NMec. Cathepsin L activity is difficult to distinguish from that of cathepsin B.

However, unlike cathepsin B, cathepsin L is capable of initiating hydrolysis of a variety of soluble and insoluble protein substrates (Yamashita and Konagaya, 1990b). Cathepsin L has been reported to be the most active cysteine lysosomal protease in degrading various protein substrates, such as azocasein, casein, collagen, elastin, histones, insulin, hemoglobin, and myofibrillar proteins (An et al., 1994b; Kirschke et al., 1977, 1982; Koga et al., 1990; Okitani et al., 1980).

SDS-PAGE analysis of the DEAE fractions of P-I revealed the presence of low M_r components (9700 and 14 500) which were copurified along with the M_r 28 800 polypeptide and were subsequently removed by low-pH treatment combined with SEC-HPLC. Yamashita and Konagaya (1992) reported the presence of a protease inhibitor in salmon of M_r 15 000 which copurified with cathepsin L. Furthermore, they reported that cathepsin L existed in two forms of M_r 30 000 and 37 000. Mason et al. (1985) reported human liver cathepsin L was composed of two polypeptides that were disulfide-linked: a heavy chain of 24 000, which contains the active site, and a light chain of 5000. Cathepsins L of rat and rabbit liver were reported to have similar structural characteristics (Kirschke et al., 1982, 1984; Mason et al., 1984). Assuming the observed M_r 14 500 component is an inhibitor, it is possible that the remaining low M_r (9700) peptide is associated with the large component of M_r 28 800. Perhaps the function of the putative light chain is for stabilization of the active enzyme. This would account for the loss of stability in P-I observed after low-pH treatment which dissociates low M_r components. The P-II DEAE fraction, which had no low M_r components associated with it, was also very unstable.

Cathepsin L has been purified from human liver and kidney (Baricos et al., 1988; Mason and Barrett, 1984), rat liver and brain (Kirschke et al., 1977; Marks and Berg, 1987; Towatari et al., 1978), rabbit liver and skeletal muscle (Mason et al., 1984; Okitani et al., 1980), and chicken liver (Dofour et al., 1987; Wada et al., 1987). However, very little work has been done with cathepsin L from nonmammalian species. Yamashita and Konagaya (1990a,c) purified cathepsins L and B from the white muscle of chum salmon (*Oncorhynchus keta*) during spawning migration. Salmon cathepsin L showed a much higher activity than other lysosomal proteases, including cathepsin B, in degrading the various myofibrillar and connective tissue proteins (Yamashita and Konagaya, 1990b). It was also reported to be a major protease contributing to the increased proteolytic activity in the salmon muscle leading to the extensive softening.

Cathepsin L, present in lysosomes of macrophages, is expressed at higher levels as a response to infection or infestation (Etherington, 1984). There are numerous reports on higher proteolytic activity in fish infected with Myxosporidian parasites (Konagaya and Aoki, 1981; Kudo et al., 1987; Patashnik et al., 1982). Our results suggest that the observed increase in proteolytic activity in these cases may be due to an increase in activity and/or expression of cathepsin L. The severe textural degradation often observed with parasitized Pacific whiting thus seems to be related to endogenous protease activities and, in particular, to increased levels of cathepsin L activity as induced by a pathological condition.

SUMMARY AND CONCLUSION

A protease with a maximum activity at 55 °C and pH 5.5, which has been implicated in texture degradation of fish fillets and surimi gels of Pacific whiting, was purified and shown to be cathepsin L. Two forms of cathepsin L were purified, both of which contained a polypeptide of M_r 28 800. The severe textural degradation often observed with parasitized Pacific whiting seems to be related to endogenous protease activities of fish fillets and, particularly, due to increased levels of cathepsin L activity as induced by a pathological condition.

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