Isolation and Activation of Cathepsin L–Inhibitor Complex from Pacific Whiting (*Merluccius productus*)

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Cathepsin L from Pacific whiting was separated into two activity peaks (P-I and P-II) on butyl-Sepharose. Acidification of the fractions at pH 3.3 resulted in an 11-fold increase in activity of P-I and a slight decrease in P-II on Z-Phe-Arg-NMec, suggesting that P-I is complex-formed with an inhibitor while P-II is the free enzyme. Analysis of further purified DEAE P-I fractions showed that only 55% of the activity was titrated by E-64 while low-pH-treated fractions were completely titrated. The DEAE P-I fractions showed highest pH stability at pH 4, while acidified DEAE P-I fractions showed the highest stability at pH 7.0.

Keywords: *Cathepsin L; complex; inhibitor; Pacific whiting*

INTRODUCTION

Pacific whiting muscle tissue, infected with myxosporidia (*Kudoa paniformis*), contains high levels of proteolytic activity (Patashnik et al., 1982). The protease can hydrolyze muscle and connective tissue proteins and, in turn, induce tissue softening which affects its marketability (Kabata and Whitaker, 1985). Fillets or mince that have high proteolytic activity also produce low-quality surimi products due to degradation of myosin needed to form a surimi gel (Morrissey et al., 1993). Purification of the enzyme showed that the proteolytic activity is mainly due to cathepsin L as characterized by its chemical inhibition, activity against specific substrates, temperature, and pH profiles (Seymour et al., 1994; Toyohara, 1993).

Cathepsin L is a cysteine protease that hydrolyzes a broad range of proteins including myosin, actin, nebulin, insulin, myoglobin, glucagon, azocasein, histones, hemoglobin, and insoluble collagen (Kirschke and Barrett, 1987; Koga et al., 1990; Yamashita and Konagaya, 1991). Among the synthetic substrates, *N*-carbobenzoxy-Phe-Arg 7-amino-4-methylcoumarin (Z-Phe-Arg-NMec) was shown to be most specific for cathepsin L (Barrett and Kirschke, 1981). Seymour et al. (1994) found that cathepsin L in myxosporidia-infected Pacific whiting had a pH optimum in the range of 5.5–6.0 and a temperature optimum at 55 °C against casein and azocasein. The activity was inhibited by sulfhydryl reagents, such as E-64, leupeptin, and cystatin.

Lysosomal cysteine proteases are among the most active proteases *in vivo*, and several endogenous inhibitors have been found to control their activities in extralysosomal locations (Barrett, 1986). The most potent endogenous inhibitors reported for cysteine proteases are the cystatins. Various forms of cystatins can inhibit cysteine proteases such as ficin, papain, and cathepsins B, H, and L of the papain superfamily by the formation of tight reversible complexes (Abrahamson et al., 1986; Anastasi et al., 1983; Barrett et al., 1986a). The isolated inhibitors are structurally homologous and can be categorized into a cystatin superfamily (Barrett et al., 1986b; Rawlings and Barrett, 1990).

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The binding strength of inhibitor to the protease is indicated to be highly dependent on the pH of its environment. Yamashita and Konagaya (1992) isolated a cathepsin L–inhibitor complex from chum salmon and increased the activity 22-fold after an acid treatment at pH 3.3 for 10 min at 25 °C. The complex was composed of two proteins with molecular weights of 37 000 and 15 000. Pike et al. (1992) isolated cystatins which complexed with sheep cathepsin L at pH's above 5.5. Their results showed that molecular weights of the components were 26 000 and 14 000, respectively, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Mason et al. (1985) observed that the "latent" form of cathepsin L accounted for 25% of the total Z-Phe-Arg-NMec hydrolysis in the standard assay. The latent form of cathepsin L was activated over 10-fold by acid treatment at pH 4.2 at 37 °C for 4 h followed by overnight incubation at 4 °C.

Purification of Pacific whiting protease (Seymour et al., 1994) has shown that fractions eluted from a hydrophobic chromatography resin, butyl-Sepharose, have two activity peaks (P-I and P-II) with an equal amount of total activities when analyzed with azocasein as a substrate. These data lead to several possibilities as to the composition of the peaks, including enzyme–inhibitor complex and free forms, isozymes, or heavy and light chains. The objective of this study was to characterize the identity of P-I and P-II by analyzing its activity with Z-Phe-Arg-NMec.

MATERIALS AND METHODS

Chemicals. Sodium phosphate, sodium citrate, sodium azide, Trizma base, 2-mercaptoethanol (βME), azocasein, EDTA, dithiothreitol, iodoacetic acid, Z-Phe-Arg-NMec, and ammonium sulfate were all purchased from Sigma Chemical Co. (St. Louis, MO). The butyl-Sepharose 4B resin was purchased from Pharmacia Biotechnology (Uppsala, Sweden), and DEAE Biogel A was from Bio-Rad (Richmond, CA).

Purification of Cathepsin L–Inhibitor Complex. Approximately 400 g of parasitized Pacific whiting fillets was finely chopped and centrifuged at 5000*g* for 30 min with a Sorvall centrifuge (DuPont Co., Newtown, CT) to prepare sarcoplasmic fluid. The resulting supernatant, approximately 90 mL, was then combined with an equal volume of McIlvaine's buffer (0.2 M sodium phosphate and 0.1 M citrate), pH 5.5. This mixture was heat-treated at 60 °C for 3 min in 50 mL aliquots and centrifuged at 7000*g* for 15 min using a Sorvall Model SS-34 rotor refrigerated centrifuge

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(DuPont Co., Newtown, CT). The resulting supernatant was dialyzed overnight in 20 volumes of 20 mM Tris buffer (pH 7.5), containing 10 mM sodium azide and 10 mM βME (buffer A) with two changes. After dialysis, the sample was adjusted to 1 M ammonium sulfate and loaded onto a butyl-Sepharose column which was equilibrated with 1 M ammonium sulfate in 20 mM Tris (pH 7.5), containing 10 mM sodium azide and 10 mM βME (buffer B). After the sample loading, the column was washed with buffer B until A_{280} reading was below 0.05. Elution of the column was carried out with buffer A, and volumes were collected as approximately 5 mL fractions. The fractions were assayed and pooled for proteolytic activity and protein content estimated by A_{280}.

The P-I fraction was further purified by DEAE Bio-Gel A chromatography. Fraction pools were dialyzed against 20 mM Tris, pH 7.5, at 4 °C. Samples were applied at 4 °C to a DEAE Bio-Gel A column (2.6 × 10 cm) previously equilibrated with buffer A. After washing overnight with buffer A until the A_{280} reading was less than 0.05, active fractions were eluted with a linear gradient (500 mL) of 0–300 mM NaCl. The fractions were analyzed and pooled for proteolytic activity assay and protein content on the basis of A_{280}. The fraction pool was then concentrated by ultrafiltration using a Centri-Prep 10 cartridge (Amicon, W. R. Grace & Co.-Conn., Beverly, MA) at 4 °C. Concentrated fractions of DEAE P-I were free of cathepsin L activity as assayed with Z-Phe-Arg-NMec. The fraction concentrates were acidified at pH 3.3 and used for active site titration and pH stability studies.

Proteolytic Activity Assay. The butyl-Sepharose and DEAE fractions were assayed for proteolytic activity by TCA assay (An et al., 1994). Each fraction was assayed using 2 mg of azocasein with 625 μL of MacIlvaine's buffer (pH 5.5) and 25 μL of the fraction solution, with the total volume brought up to 1.25 mL with water. The mixture was incubated for 10 min at 55 °C. Absorbance of the azo dye released from the reaction was enhanced by the addition of 10 N NaOH and was read at 428 nm.

Cathepsin L Activity Assay. The Z-Phe-Arg-NMec assay was performed according to Barrett and Kirschke (1981) with a minor modification. Butyl-Sepharose or DEAE fractions and their counterparts (5 μL) treated at pH 3.3 were diluted to 500 μL with 0.1% Brij 35 and preincubated for 1 min in 250 μL of assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA, and 8 mM dithiothreitol, pH 5.5). The enzymatic reaction was performed for 3 min at 30 °C by the addition of 250 μL of 20 μM Z-Phe-Arg-NMec. To stop the reaction, 200 μL of 5 mM iodoacetic acid was added to the mixture, and fluorescence was read at an excitation wavelength of 370 nm and an emission wavelength of 460 nm with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD). No interfering activity on Z-Phe-Arg-NMec was observed, and the blank readings were negligible. The unit of activity was defined as micromoles of methylcoumarin released per minute.

Acidification. Butyl-Sepharose or DEAE fractions were acidified by adding 50 μL of each fraction to 100 μL of 0.05 M sodium citrate–0.05 M phosphate buffer (pH 3.3) containing 1 mM EDTA and 1 mM βME. The mixture was allowed to incubate for 10 min at 20 °C prior to the Z-Phe-Arg-NMec assay.

Active Site Titration. Active site concentration of the enzyme was determined by titration with E-64 by the method described by Barrett and Kirschke (1981). Acidified and nonacidified DEAE P-I fractions, 25 μL, were preincubated with 25 μL of 25–300 nM E-64 and 50 μL of the modified MacIlvaine's buffer (0.2 M sodium phosphate, 0.1 M citrate, 4 mM of disodium EDTA, and 8 mM dithiothreitol), pH 7.0, at 30 °C for 5 min followed by dilution with 0.1% Brij 35 to the total volume of 550 μL. The mixture was then analyzed for activity with Z-Phe-Arg-NMec for 3 min at 30 °C as described under Cathepsin L Activity Assay. Molarity of the enzyme active sites was determined by a linear plot of activity against E-64 molar concentrations, as described by Barrett and Kirschke (1981).

pH Effect. DEAE fraction concentrates and their acidified counterparts (approximately 7 pmol, as determined by active site titration) were diluted to 50 μL with 0.1% Brij 35. The diluent was added with equal volume of the modified MacIlvaine's buffer, pH 3–9, and preincubated for 30 min at 0 °C. The enzyme mixture was then analyzed for activity by adding an assay cocktail containing 450 μL of 0.1% Brij 35, 200 μL of sodium acetate buffer, pH 5.5, and 250 μL of 20 μM Z-Phe-Arg-NMec prewarmed to 30 °C. The activity was measured by reaction for 3 min at 30 °C as described under Cathepsin L Activity Assay.

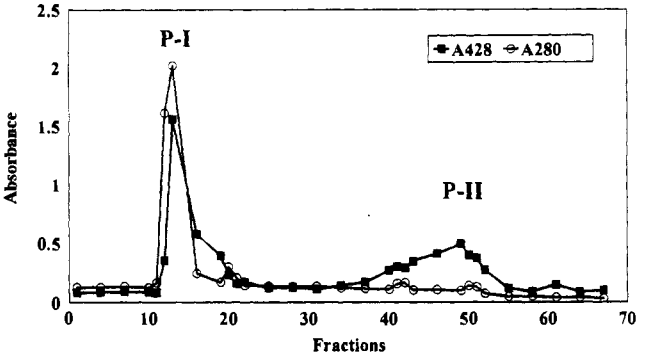

Figure 1. Elution profile for butyl-Sepharose fractions. Activity was determined by TCA-azo assay and expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

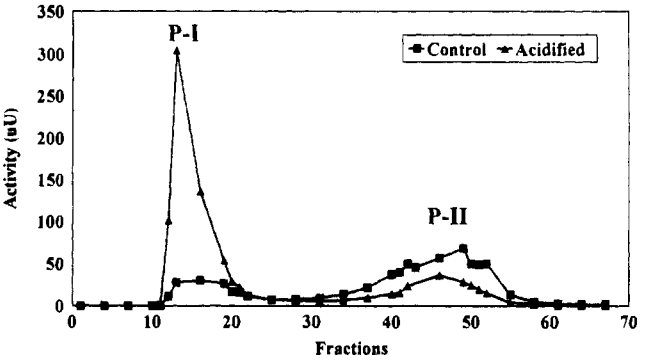

Figure 2. Activity of butyl-Sepharose fractions on Z-Phe-Arg-NMec with or without acidification prior to activity assays.

RESULTS AND DISCUSSION

Pacific whiting muscle tissue extracts yielded two activity peaks (P-I and P-II) by hydrophobic column chromatography using the azocasein assay for protease detection (Figure 1). P-II was relatively pure, and P-I had more contaminating proteins coeluting with the peak, as shown by A_{280} readings. Both of the peaks were capable of hydrolyzing Z-Phe-Arg-NMec (Figure 2). Acidification of the butyl-Sepharose fractions resulted in a maximum 11-fold increase in activity of P-I fractions and a slight decrease in the activity of P-II when using Z-Phe-Arg-NMec as a synthetic substrate (Figure 2). Yamashita and Konagaya (1992) showed a similar effect with the enzyme–inhibitor complex of cathepsin L in chum salmon. An acid treatment at pH 3.3 dissociated the cathepsin L–inhibitor complex, resulting in a 22-fold increase in activity, and the enzyme and inhibitor recombined when the pH was above 4.0. Pike et al. (1992) also found that pH 5.5 and above was necessary for formation of the sheep liver cathepsin L–cystatin complexes. The pH of buffer used in this study to elute cathepsin L from butyl-Sepharose resin was 7.5, which would allow formation of an enzyme–inhibitor complex. The observed increase in activity of P-I suggests that an inhibitor is dissociating from the enzyme at the low pH. No increase in activity was observed in P-II after the acid treatment, indicating no

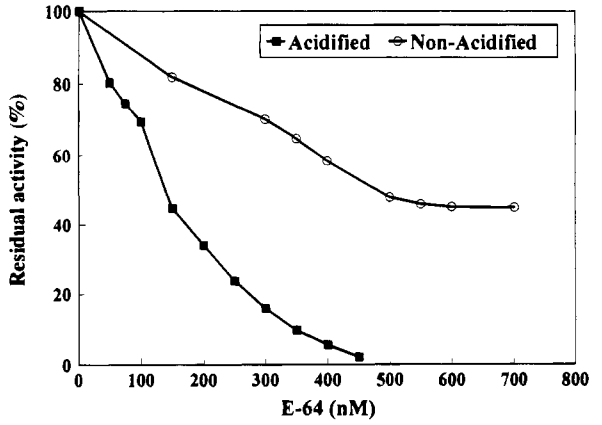

Figure 3. Active site titration of P-I fraction further purified on DEAE. The DEAE P-I fraction was acidified prior to cathepsin L activity assay using Z-Phe-Arg-NMec.

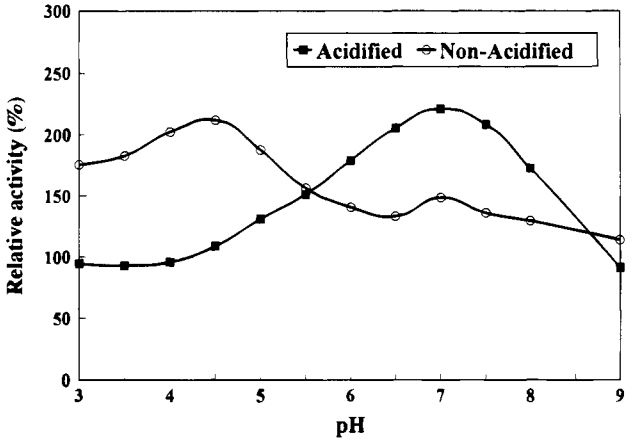

Figure 4. Effect of pH on acidified and nonacidified P-I fractions further purified on DEAE. The protease was incubated at various pH conditions for 30 min at 0 °C prior to cathepsin L activity assay using Z-Phe-Arg-NMec.

complex formation of P-II with inhibitor(s). The cathepsin L–inhibitor complex form appears to be less hydrophobic than the free enzyme, since P-I elutes from the butyl-Sepharose resin prior to P-II.

Active site titration of DEAE P-I fractions showed a typical behavior of protease–inhibitor complex. Approximately 55% of the total activity was titrated by E-64, while 45% retained activity on Z-Phe-Arg-NMec (Figure 3). However, acidification of DEAE P-I prior to treatment with E-64 transformed the remaining activity susceptible to E-64 titration, and 98% of its activity was titrated (Figure 3). Previously, Pike et al. (1992) reported a similar result with isolated cystatin-complexed cathepsin L in which E-64 titrated only 60% of the total activity. The authors proposed two different types of cathepsin L–cystatin binding modes, i.e., normal and covalent binding, and suggested that the remaining 40% of nontitratable activity was due to the dissociation of the normal enzyme–cystatin complex. Furthermore, acidification of Pacific whiting cathepsin L had a significant effect on pH stability. Two different pH ranges were observed for the maximal stabilities of acidified and nonacidified DEAE P-I fractions (Figure 4). Acidified DEAE P-I, postulated to be relatively free of inhibitors, showed the highest pH stability at pH 7.0, while nonacidified fractions containing enzyme–inhibitor complexes showed the maximum activity peak at pH 4.5 followed by a minor activity peak at pH 7.0. The maximum activity at pH 4.5 found with nonacidified P-I may reflect the combinatorial effects of the enzyme activity at that pH and the maximal dissociation of enzyme–inhibitor complexes to expose the active sites to the substrates. Therefore, the pH activity profile observed with acidified DEAE P-I is considered to reflect the true pH-stability characteristics of Pacific whiting cathepsin L.

Cystatins are tight binding reversible inhibitors of cysteine proteases and are not known to inhibit proteases from other catalytic classes (Salvesen and Nagase, 1989). Cathepsin L has been reported to strongly bind cystatin with a K_i value of 3×10^{-12}, lower than those of papain and cathepsins B and H (Anastasi et al., 1983). The formation of a complex with inhibitor can alter isoelectric points (pI) of pure enzymes. Cystatin showed complete incorporation of protein into complexes with papain, and the complex migrated between papain and cystatin bands in the isoelectric focusing gel (Anastasi et al., 1983). In our study, the p*I* values of P-I complex and P-II of Pacific whiting were found to be 5.6 and 4.9, respectively (Seymour and An, 1993). However, after the acid treatment to dissociate the complex, the p*I* values of P-I and P-II were shown to be the same, 4.9 (Seymour et al., 1994).

Our results suggest that P-I and P-II may be the same enzyme in complex and free forms, both containing cathepsin L activity. We previously observed a contaminating protein copurifying with Pacific whiting cathepsin L on SDS–PAGE (Seymour et al., 1994). It is now postulated that this protein band is due to the endogenous inhibitor. Mason et al. (1985) reported that acid treatment was the most important step in the purification scheme of cathepsin L from human liver. These facts indicate that effective purification of cathepsin L from Pacific whiting should include an acidification step to dissociate the enzyme–inhibitor complexes.

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