Characterization of the Proteases Involved in Gel Weakening of Beef Heart Surimi†

Baowu Wang and Youling L. Xiong*

Department of Animal Sciences, University of Kentucky, Lexington, Kentucky 40546

The goal of this study was to elucidate the nature and characteristics of the proteases involved in gel weakening of beef heart surimi. Acidic (E1) and neutral (E2) protease extracts were prepared from the surimi. The major active components in E1 were found to be cathepsins B and L. E1 exhibited optimum activity to hydrolyze substrates specific to cathepsins B and B+L at 50 °C and pH 5.5. At pH 6.0, it retained ∼50% of its maximum activity. The catheptic activity of E1 was inhibited almost completely by E-64 and leupeptin. The active component in E2 was unidentified and was not inhibited by cysteine or serine protease inhibitors. However, beef plasma powder effectively inhibited the hydrolysis of FITC-casein and myosin heavy chain by E2.

Keywords: *Beef heart; surimi; cathepsin; protease; protease inhibitor*

INTRODUCTION

Gel weakening, commonly known as modori, occurs in many fish surimi during thermal processing (Lanier et al., 1981; Nagahisa et al., 1981; Miller and Spinelli, 1982; Chang-Lee et al., 1990; Morrissey et al., 1993). When minces or surimi from these fish are heated slowly through the 60−70 °C temperature range, a reduction in gel strength can be observed. The primary cause for this temperature-dependent gel weakening is believed to be certain enzymes that hydrolyze myofibrillar proteins, especially myosin, into small fragments (Wasson, 1992). However, there is no consensus on the nature and specific source of enzymes involved in the gel weakening of fish surimi (Wasson, 1992). Some researchers ruled out cathepsins, particularly cathepsins A, B, C, and D, because these cathepsins cannot hydrolyze myofibrillar proteins above pH 7.0 which is the typical pH of fish surimi (Mackinodan et al., 1982; Erickson et al., 1983). On the other hand, some experimental evidence points to heat-stable neutral and alkaline protease(s) as the possible causing agents for gel weakening of certain fish surimi (Su et al., 1981a,b; Busconi et al., 1984; Mackinodan et al., 1985). These proteases may include both serine and cysteine proteases, that is, proteases that contain serine and cysteinal residues at the catalytic center (Toyohara, 1990). Despite the controversy on the exact mechanism for modori, a series of recent studies have demonstrated that cathepsins B and L are responsible for gel softening of fish surimi prepared from Pacific whiting and mackerel (An et al., 1994; Jiang et al., 1994, 1996, 1997; Seymour et al., 1994). Cathepsins B and L from these fish were shown to hydrolyze myofibrillar proteins at pH 6.5−7.5, indicating a strong likelihood of their involvement in gel weakening of some fish surimi (Jiang et al., 1997).

Although the modori phenomenon has been studied extensively in fish surimi, it has not been regarded as a particular problem for mammalian or avian meat products (Young et al., 1992). However, recent discoveries of a similar gel-softening phenomenon in surimi-like materials prepared from low-grade meat or meat byproducts have pointed to the necessity to look into the possible role of muscle endogenous proteases. Park et al. (1996) observed gel weakening in surimi-like material prepared from beef and pork when heated at 50 °C. In a recent study, we found a similar temperature-dependent gel weakening in surimi-like material produced from beef cardiac muscle (Wang and Xiong, 1998). Our preliminary results indicated that proteolytic enzymes were involved, but the nature and characteristics of the enzymes have not been determined. The objectives of the present study were to identify and characterize the endogenous proteases that were responsible for textural changes in cooked beef heart surimi gel and to find effective inhibitors against these proteases.

MATERIALS AND METHODS

Materials. Fluorescein isothiocyanate-labeled casein (FITC-casein), fluorescein isothiocyanate (FITC), 7-amino-4-methylcoumarin, N-CBZ-Phe-Arg-7-amido-4-methylcoumarin, N-CBZ-Arg-Arg-7-amido-4-methylcoumarin, L-Arg-7-amido-4-methylcoumarin, phenylmethanesulfonyl fluoride (PMSF), leupeptin, and L-*trans*-epoxysuccinylleucylamide (4-guanidino)butane (E-64) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine plasma powder (BPP) was obtained from AMPC Inc. (Ames, IA). Soy protein isolate (SPI) was obtained from Protein Technologies International (St. Louis, MO). Fresh eggs were purchased from a local grocery store, and whole egg white (EW) was separated from yolk prior to use.

Preparation of Beef Heart Surimi (BHS). The method described previously (Wang et al., 1997) was followed to prepare BHS. Briefly, lean beef heart muscle was ground through a 3 mm plate, mixed with 0.02% propyl gallate (based on muscle weight), and washed three times. Distilled water was used for the first two washes (meat/water ratio 1:10). Sodium phosphate buffer (25 mM, pH 6.0, meat/buffer ratio 1:5) was used for the third wash. The mixture was blended using a Waring blender at high speed for 1 min, adjusted to

* Author to whom correspondence should be addressed [telephone (606) 257-3822; fax (606) 257-5318; e-mail ylxiong@pop.uky.edu].

† Approved for publication as journal article 98-07-122 by the Director of the University of Kentucky Agricultural Experiment Station.

10.1021/jf980845i CCC: $18.00
Published on Web 02/05/1999

pH 6.0, and centrifuged at 3000*g* for 15 min. The pellet was collected as BHS, kept on ice (0 °C), and used within 2 days.

Preparation of Crude Enzyme Extracts. A crude cathepsin extract (E1) was prepared according to the method of Porter et al. (1996). BHS was blended with 3 volumes of extraction buffer (20 mM sodium acetate, 1% NaCl, 0.02% NaN_3, pH 5.80) using a Waring blender at high speed for 2 min. The homogenate was set at 4 °C for 1 h and then centrifuged at 10000*g* for 20 min (4 °C). The supernatant was filtered through glass wool to remove fat particles. The filtrate, designated E1, was used for the cathepsin activity assay.

A crude neutral protease extract (E2) was prepared according to the method of Rosell et al. (1996). BHS was homogenized with 3 volumes of extraction buffer (50 mM Tris, 3 mM EDTA, 10 mM mercaptoethanol, pH 8.3) using a Waring blender at high speed for 2 min. The homogenate was set at 4 °C for 1 h and then centrifuged at 28000*g* for 30 min (4 °C). The supernatant was filtered through glass wool to remove fat particles. The filtered supernatant was adjusted to pH 7.5 and centrifuged at the same condition again. The latter supernatant, designated E2, was used for the neutral protease activity assay.

Assay Procedures. The activities of cathepsins B and L were measured according to the method of Barrett and Kirchke (1981). N-CBZ-Arg-Arg-7-amido-4-methylcoumarin and N-CBZ-Phe-Arg-7-amido-4-methylcoumarin were used as specific substrates for cathepsin B and cathepsin B+L, respectively. The activity of cathepsin H was measured according to the method of Lee et al. (1996) using L-Arg-7-amido-4-methylcoumarin as the substrate. The following assay procedures, which were the same for all three cathepsins, were used. An aliquot of 500 μL of E1 (diluted to 3 mg/mL protein with 0.1% Brij 35) was mixed with 250 μL of assay buffer at pH 5.5 (340 mM sodium acetate, 60 mM acetic acid, 4 mM EDTA, and 8 mM dithiothreitol). The mixture was equilibrated in a water bath set at specific temperature (30−80 °C) for 1 min, and then 250 μL of substrate solution (20 μM) was added. After 10 min of incubation at the same temperature, the reaction was stopped by adding 1 mL of stopping reagent (100 mM sodium monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3). The mixture was centrifuged at 3000*g* for 15 min, and fluorescence intensity (excitation, 370 nm; emission, 460 nm) of the supernatant was measured. The spectrofluorometer (Fluoro IV, Gilford, Oberlin, OH) was calibrated to 100% using standard solution (0.5 μM 7-amino-4-methylcoumarin). A blank was run parallel to the samples except that E1 was added at the end of incubation.

The activity of E2 was measured according to the method of Rosell et al. (1996) using FITC-casein as the substrate. An aliquot of 300 μL of E2 (diluted to 3 mg/mL protein) was mixed with 100 μL of FITC-casein (2 mg/mL in 100 mM Tris-acetate, 10 mM mercaptoethanol, pH 7.5). After 10 min, the reaction was stopped by adding 0.5 mL of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 3000*g* for 15 min. An aliquot of 250 μL of supernatant was mixed with 1.5 mL of sodium phosphate buffer (500 mM, pH 8.5). The fluorescence intensity (excitation, 490 nm; emission, 513 nm) was measured. The spectrofluorometer was calibrated to 100% using 20 ng/tube FITC. A blank was run with E2 and 0.5% TCA being added at the end of incubation.

Effects of Protease Inhibitors against E1 and E2. The following protease inhibitors were prepared: E-64 (20 μg/mL), leupeptin (L, 20 μg/mL), PMSF (1.74 mg/mL), BPP (52 mg/mL), egg white (EW, 532 mg/mL), and soy protein isolate (SPI, 44 mg/mL). Aliquots of 0.1 mL of these solutions were mixed with 0.4 mL of E1 (3 mg of protein/mL) or 0.3 mL of E2 (3 mg of protein/mL) and incubated at room temperature for 2 min. This would ensure that proteases from E1 and E2 were already reacted with the inhibitors before mixing with their respective substrates in the subsequent enzyme assay (which was performed using the same procedures as described above).

Hydrolysis of Crude Myosin by E1 and E2. To verify that E1 and E2 had the ability to hydrolyze myosin, crude myosin was isolated from prerigor beef heart muscle (within 30 min of exsanguination) without further purification (Margossian and Lowey, 1982). Prior to use, the crude myosin was dialyzed (0.6 M NaCl, 50 mM sodium phosphate, pH 6.0) for 12 h with the dialyzing solution being replaced every 3 h. Crude myosin was incubated with E1 and E2 at pH 6.0 (0.2 M sodium phosphate, 0.1 M citric acid, 4 mM EDTA) at 50 °C for 2 h. A blank control was incubated in parallel with the samples without added E1 or E2. In other control samples, protease inhibitors were also added as described above to see if they can prevent E1 or E2 from hydrolyzing myosin.

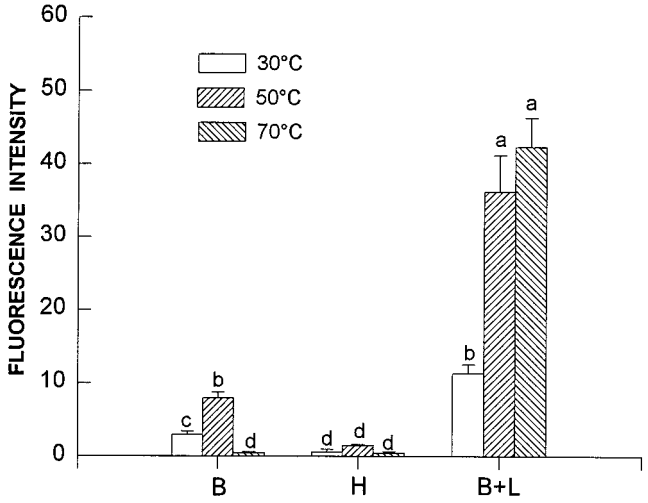

Figure 1. Activities of capthepsins B, H, and B+L in E1 at pH 5.5 and 30, 50, and 70 °C. The assay was conducted for 10 min. (Bars with different letters are significantly different, $P \leq 0.05$.)

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Myosin samples treated with enzymes and inhibitors were analyzed with SDS−PAGE according to the method of Laemmli (1970) using an SE 250 Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). The stacking gel and the resolving gel contained 3 and 10% acrylamide, respectively. Samples for electrophoresis were prepared by dissolving the incubated mixture with 5% SDS containing 0.1% 2-mercaptoethanol. After incubating at 80 °C for 1 h to completely dissolve the proteins, the samples were centrifuged at 5000*g* for 15 min. The supernatant was diluted to 2 mg/mL protein determined using the biuret method (Gornall, 1949). To eliminate the interference of SDS, the solution was centrifuged at 2000*g* for 5 min before the absorbance at 540 nm was read. The above solution was mixed with sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.125 M Tris, pH 6.8) at a 1:1 ratio. A 20 μL sample was loaded to each well of the polyacrylamide gel.

Statistical Analysis. The experiment was a randomized complete block design. The blocking factor was the replicates, that is, the repeated experiments run at different days. Each test was repeated at least two times. Overall *F* test was done using linear models of general AOV with the Statistix 3.5 software package (Analytical Software, Inc., St. Paul, MN) for microcomputers. Differences between means were analyzed using least significant difference (Snedecor and Cochran, 1989). The level of difference was defined at $P \leq 0.05$.

RESULTS AND DISCUSSION

Crude Cathepsin Extract, E1. *Active Proteases in E1.* Three different synthetic peptides specific to cathepsins B, H, and B+L were incubated with E1. Hydrolysis of these synthetic peptides would release the labeled fluorescent agent, making fluorescence intensity a sensitive indicator for the activity of these cathepsins. As shown in Figure 1, cathepsin H activity was only minimal, but activities of cathepsin B and cathepsins B+L were significantly higher ($P < 0.05$) at 50 °C

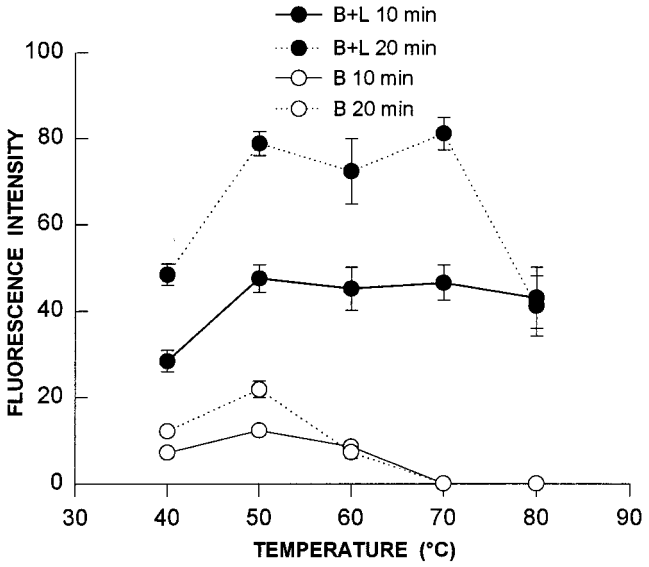

Figure 2. Activities of cathepsins B and B+L in E1 at pH 5.5 as a function of incubating temperature and time.

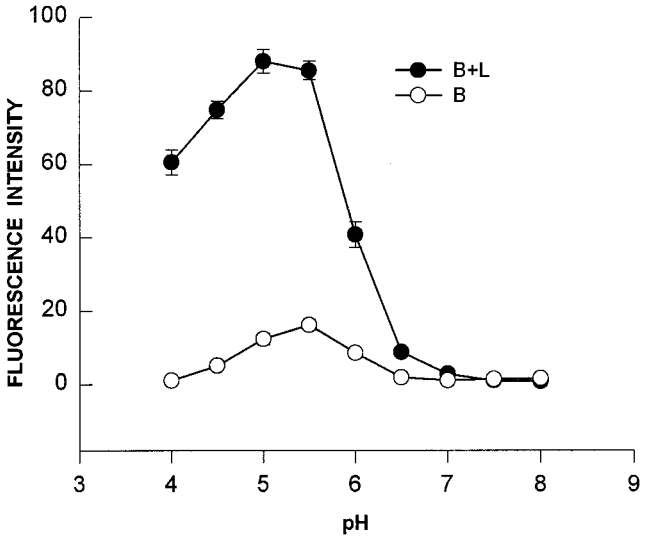

Figure 3. Activities of cathepsins B and B+L in E1 as a function of pH. The enzyme assay was conducted at 50 °C for 20 min.

incubation. This result indicated that the major active cathepsins in E1 were cathepsins B and L, especially cathepsin L. Considering its low activity in E1, cathepsin H would unlikely play a significant role in the hydrolysis of myofibrillar proteins during the BHS cooking process. Therefore, later experiments focused only on cathepsins B and B+L.

Activities of Cathepsins B and L in E1 as a Function of Temperature and pH. E1 exhibited a low activity to hydrolyze the substrate specific to cathepsin B in a narrow temperature range (around 50 °C) but a high activity to hydrolyze the substrate specific to cathepsin B+L in a broad temperature range (50−70 °C) (Figure 2). As the incubation time was prolonged from 10 to 20 min, the extent of hydrolysis (fluorescence intensity) of the cathepsin B's substrate increased slightly (T < 60 °C, by 5−10 units), but that of cathepsin B+L's substrate increased substantially (by 20−35 units) over the temperature range of 40−70 °C. The results indicated that cathepsin L was more heat-resistant than cathepsin B and would seem to play a more important role than cathepsin B in hydrolyzing myofibrillar proteins during cooking of BHS. This result was verified by thermal treatment of E1 at 70 °C for 20 min before analysis of its cathepsins B and L activities. After this heat treatment, E1 lost its activity to hydrolyze cathepsin B's substrate, but retained a high activity to hydrolyze cathepsin B+L's substrate (data not presented). Because a major reduction in BHS gel strength occurred at 50 °C (Wang and Xiong, 1998), this suggests that the gel softening is related to both cathepsins. Because cathepsin B and cathepsin B+L were assayed using different substrates, their relative activities could not be differentiated in this study.

Although active over a broad pH range, cathepsins B and L in the E1 enzyme extract exhibited their greatest activity between pH 5.0 and 5.5 (Figure 3). Note that at pH 6.0, the optimal gelation pH for BHS (Xiong et al., 1993), E1 retained ~50% of its maximum catalytic activity. Therefore, both lysosomal proteases present in E1 could hydrolyze myofibrillar proteins, thereby reducing the protein gel matrix formation at pH 6.0 (Wang and Xiong, 1998).

Effects of Protease Inhibitors against E1 Enzymes. Except PMSF, other synthetic protease inhibitors effectively reduced the activities of cathepsins B and B+L (P < 0.05) (Figures 4 and 5). The proteinaceous food ingredients EW, BPP, and SPI also exhibited significant inhibitory effects (P < 0.05). Although PMSF inhibited the hydrolysis of substrate specific to cathepsin B (P < 0.05) (Figure 5), it did not exhibit significant effects on the hydrolysis of substrate specific to cathepsin B+L (P > 0.05) (Figure 4), indicating that PMSF was not an effective inhibitor against cathepsin L in E1.

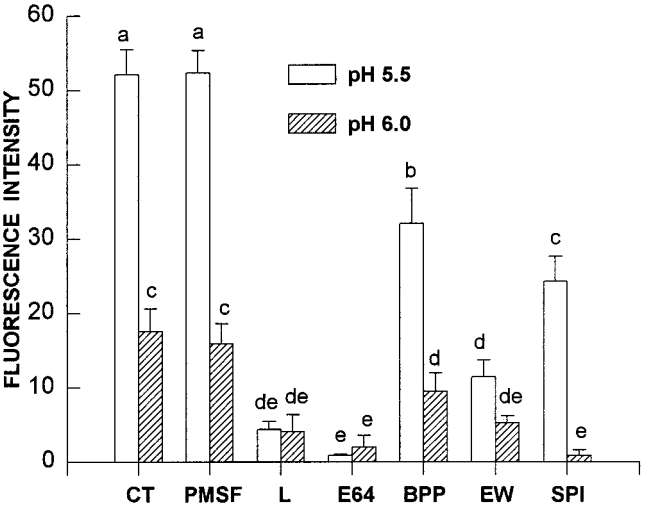

Figure 4. Effects of protease inhibitors on the hydrolysis of the substrate specific to cathepsin B+L in E1 at 50 °C, pH 5.5 or 6.0, for 10 min. CT, control; PMSF, phenylmethanesulfonyl fluoride; L, leupeptin; E-64, epoxysuccinylleucylamide (4-guanidino)butane; BPP, beef plasma powder; EW, fresh egg white; SPI, soy protein isolate. (Bars with different letters are significantly different, $P \leq 0.05$.)

Protease inhibitors can be used to identify the nature of proteases. Some of them are specific inhibitors, and others may have multiple functions or contain multiple active components. For instance, E-64 is a specific cysteine protease inhibitor; leupeptin is primarily effective against cysteine proteases, but it is also effective against some serine proteases; PMSF is primarily effective against serine proteases, but it is also effective against some cysteine proteases; and EDTA is an effective inhibitor against calpains nonspecifically (Barrett and Kirschke, 1981; Bond and Butler, 1987).

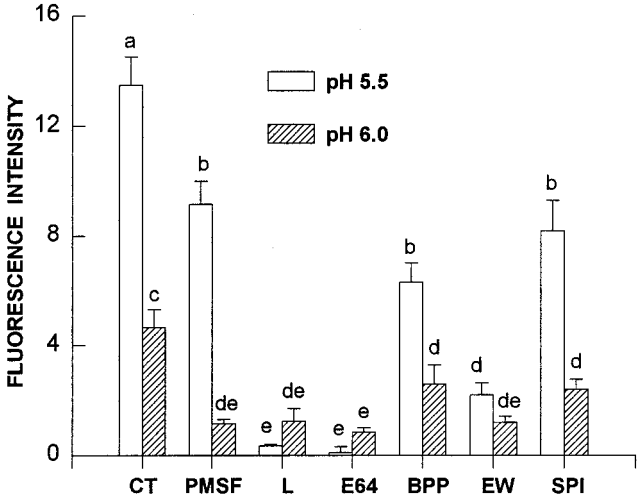

Figure 5. Effects of protease inhibitors on the activity of cathepsin B in E1 incubated at 50 °C, pH 5.5 or 6.0, for 10 min. For abbreviations, see Figure 4. (Bars with different letters are significantly different, $P \leq 0.05$.)

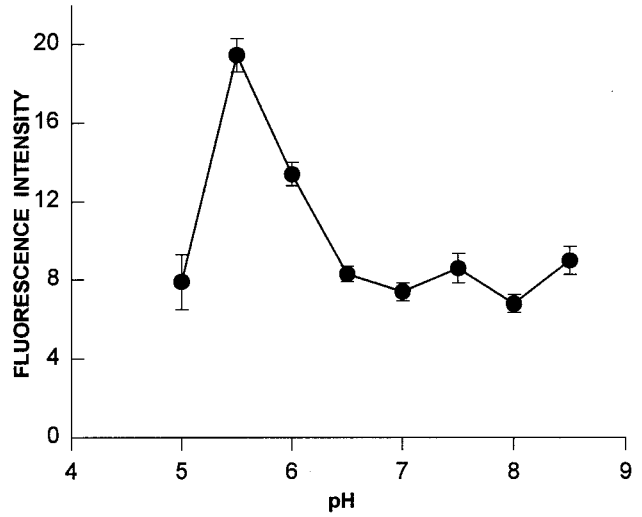

Figure 6. Effect of pH on the hydrolysis of FITC-casein by E2 at 60 °C for 10 min. The E2 extract (3.0 mg/mL) was diluted using buffers of different pH values at a 1:1 ratio to a protein concentration of 1.5 mg/mL.

Empirical evidence demonstrated that BPP, EW, and potato extract had various degrees of protease inhibition in surimi processing (Hamann et al., 1990; Akazawa et al., 1993; Porter et al., 1993; Reppond and Babbitt, 1993), but the active enzyme inhibitory components in these food ingredients have not been identified (Weerasinghe et al., 1996). Results from this study demonstrated that cathepsin B-like and cathepsin L-like proteases in E1 could be inhibited by BPP, EW, and SPI. Therefore, these food grade ingredients have the potential for enhancing the gelation of beef heart surimi during thermal processing.

Crude Neutral Protease Extract, E2. *Activity of E2 at Different pH Values and Temperatures.* The optimum temperature for E2 against the synthetic substrate FITC-casein was 60 °C, and it exhibited only minimal activity above 65 °C (data not presented). Its optimum pH was ~5.5, but it retained ~50% of its maximum activity in the pH range of 5.0−8.5 (Figure 6). On the basis of its pH profile, the major active protease in E2 was unlikely to be calpain because calpain has an optimum pH ~7.5 (Koohmaraie et al., 1990).

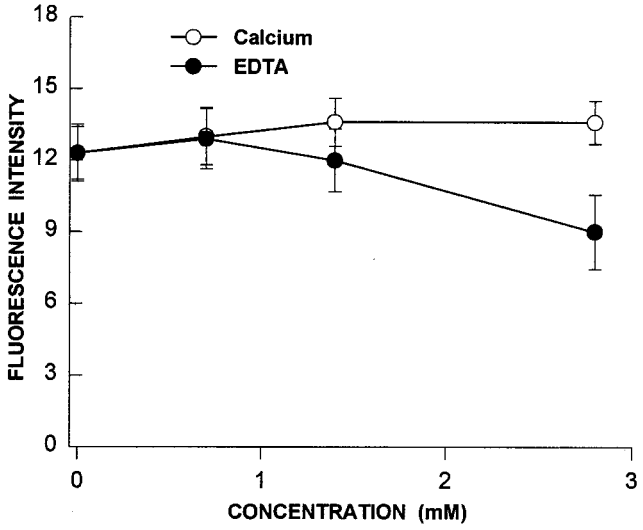

Figure 7. Effects of calcium and EDTA on the hydrolysis of FITC-casein by E2 at 60 °C, pH 7.5, for 10 min.

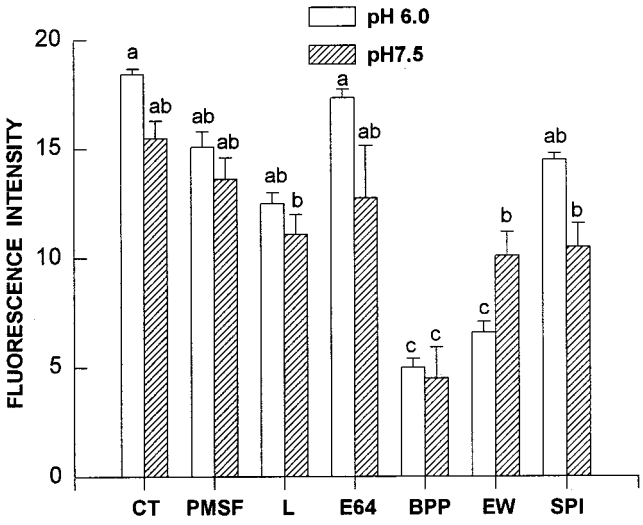

Figure 8. Effects of protease inhibitors on the hydrolysis of FITC-casein by E2 at 60 °C, pH 6.0 and 7.5, for 10 min. For abbreviations, see Figure 4. (Bars with different letters are significantly different, $P \leq 0.05$.)

Effects of Calcium and EDTA on the Activity of E2. To verify that the major active protease in E2 was not calpain, EDTA (calpain inhibitor) and calcium (calpain activator) were added to the medium to see if they had any significant impact on the activity of E2. As shown in Figure 7, E2 was not activated by the addition of calcium, nor was it significantly inhibited by the addition of EDTA. This further supported that E2 was not calpain. Furthermore, when a higer concentration of calcium was used, the activity of E2 was decreased (data not presented). This indicated that the low calpain activity was not due to insufficient concentration of calcium.

Effects of Protease Inhibitors on E2. The strongest protease inhibitor against E2 was BPP at either pH 6.0 or 7.5 (Figure 8). Note that PMSF, E-64 and leupeptin had only minimal inhibitory effects against E2, suggesting that there were minimal cysteine or serine proteases in this enzyme extract. The nature of E2 needs to be identified by further investigation.

SDS−PAGE. E1 and, to a lesser extent, E2 hydrolyzed myosin heavy chain (Figures 9 and 10). The resulting polypeptides formed a dense band with mo-

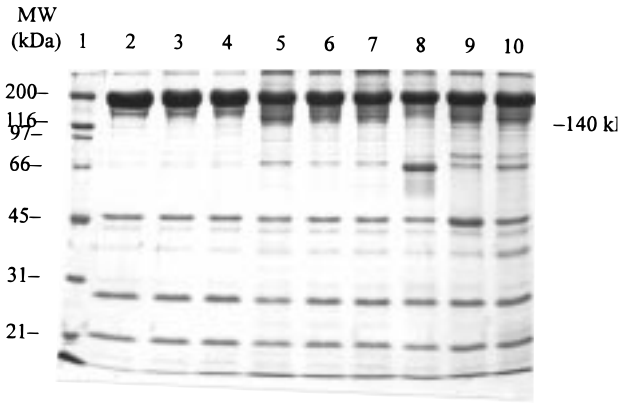

Figure 9. Degradation pattern of crude beef heart myosin incubated with E1 and examined using SDS−PAGE: (lane 1) protein standard; (lane 2) crude myosin without incubation; (lane 3) crude myosin with incubation; (lane 4) crude myosin plus E1 without incubation; (lane 5) crude myosin plus E1 with incubation; (lanes 6−10) crude myosin plus E1 and protease inhibitors E-64 (lane 6), PMSF (lane 7), BPP (lane 8), EW (lane 9), and SPI (lane 10) with incubation. Incubation conditions: 50 °C, pH 6.0, 2 h.

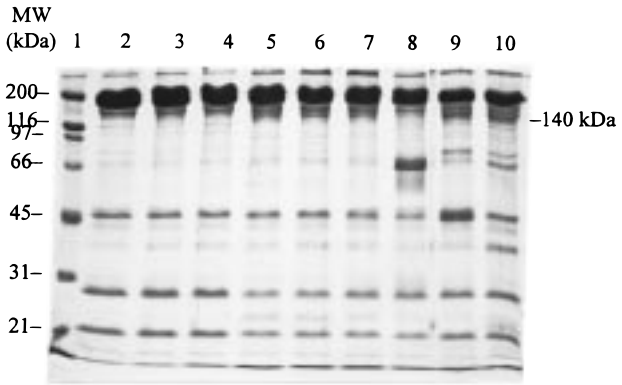

Figure 10. Degradation pattern of crude beef heart myosin incubated with E2 and examined using SDS−PAGE: (lane 1) protein standard; (lane 2) crude myosin without incubation; (lane 3) crude myosin with incubation; (lane 4) crude myosin plus E2 without incubation; (lane 5) crude myosin plus E2 with incubation; (lanes 6−10) crude myosin plus E2 and protease inhibitors E-64 (lane 6), PMSF (lane 7), BPP (lane 8), EW (lane 9), and SPI (lane 10) with incubation. Incubation conditions: 50 °C, pH 6.0, 2 h.

lecular weight around 140 kDa. Hara et al. (1988) reported that cathepsin B had the ability to hydrolyze myosin heavy chain at pH 6.0, and this hydrolytic activity was activated by NaCl. Recently, Jiang et al. (1997) found that both cathepsins B and L had the ability to hydrolyze myosin heavy chain at pH values >6.0. All of the protease inhibitors inhibited E1 to some extent, but only BPP showed pronounced inhibition against E2. Although E-64 had maximum inhibition against the hydrolysis of synthetic peptides by cathepsins in E1, it did not show equivalent inhibition on hydrolysis of myosin heavy chain with SDS−PAGE analysis. This result was unexpected but may be explained by considering three factors. First, hydrolysis of proteins by proteases cannot always be predicted on the basis of their action on synthetic substrates because the structure of substrate proteins and peptides dictates, to some extent, the type of proteolytic action exerted by a protease (Bond and Butler, 1987). In other words, proteases do not always have correspondent activity on hydrolysis of short peptides and large molecular weight proteins such as myosin heavy chain. Second, beef heart surimi may contain multiple active proteases that can hydrolyze myosin heavy chain. Even though cysteinal cathepsins have been inhibited by E-64, other proteases can still hydrolyze myosin heavy chain. Third, food grade protease inhibitors such as BPP contain multiple inhibitory components that can inhibit different proteases against the hydrolysis of myosin heavy chain (Weerasinghe et al., 1996). Their inhibitory components may work in concert to substantially reduce myosin heavy chain hydrolysis.

Implications of Protease Inhibitors. Gel weakening impairs the quality of product made from many fish surimi (Lee, 1984; Lanier, 1986). This phenomenon has tremendous species variability, and the enzymes involved come from diverse sources (Shimizu et al., 1981). There has been evidence that points to the involvement of alkaline, neutral (Toyohara et al., 1990; Wasson, 1992; Kołodziejska and Sikorski, 1996), and acidic (An et al., 1994; Jiang et al., 1997) cathepsins in the gel weakening of some fish surimi.

The results of the present study showed that BHS contained proteases that were active at both acidic and neutral pH values. In view of the fact that gel weakening is not generally considered a problem for comminuted meat products such as frankfurters, we speculate that whole meat mince may contain protease inhibitors that may prevent muscle proteins from being hydrolyzed. This may be particularly true for beef heart because it contains a large amount of residual blood and myoglobin. We assume that during surimi preparation, most protease inhibitors, including those in the plasma, were removed, but a significant amount of proteases remains in the surimi fraction. This can be substantiated by the following observations: (1) BPP enhances the gel-forming ability of fish surimi (Lee, 1984) and beef heart surimi (Wang and Xiong, 1998), and (2) myoglobin acts as a protease inhibitor against cathepsins, calpains, and other proteases (Rosell et al., 1996). It is highly possible that these water-soluble protease inhibitors which exist in the muscle are easily washed out during surimi preparation. Without these protease inhibitors, surimi-like materials become more susceptible to gel weakening than whole mammalian or avian meat.

Conclusions. The active proteases in beef heart surimi were cathepsin L, cathepsin B, and an unidentified component in the neutral enzyme extract. These proteases were involved in the hydrolysis of myosin heavy chain during incubation at 50 °C but could be inhibited by some synthetic and food grade protease inhibitors, with BPP being the most effective. The inconsistent results between SDS−PAGE and cathepsin activity may be attributed to the hydrolysis of myosin by other enzymes and the multiple actions of food grade ingredients, especially beef plasma powder.

ABBREVIATIONS USED

PMSF, phenylmethanesulfonyl fluoride; FITC, fluorescin isothiocyanate; BPP, beef plasma powder; EW, egg white; SPI, soy protein isolate; E1, crude enzyme extract 1 at pH 5.8; E2, crude enzyme extract 2 at pH 8.3.

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Received for review July 30, 1998. Revised manuscript received November 25, 1998. Accepted December 23, 1998. This research was supported by a CSRS/USDA National Research Initiative (NRI) grant, under Agreement Grant 96-35500-3324.

JF980845I