Evidence of Proteolytic Activity and Its Effect on Gelation of Myofibrillar Protein Concentrate from Bovine Cardiac Muscle

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To explore the impact of endogenous proteases on the gelation properties of beef heart surimi, the surimi samples were incubated at different temperatures. Maximum (4.8 N) and minimum (3.3 N) gel strengths were produced by surimi sols preincubated at 60 and 50 °C, respectively. Preincubation at 60 °C as well as slow linear heating also produced gels with the highest storage modulus and water-holding capacity. Proteolytic activity with an optimum pH of 5.0 and temperature close to 50 °C was discovered. Addition of protease inhibitors, particularly bovine plasma powder, reduced the extent of gel weakening caused by the proteases and, concomitantly, enhanced the water-holding capacity of the surimi gel. SDS−PAGE showed that in the absence of protease inhibitors, myosin was partially hydrolyzed and a 130 kDa polypeptide formed during preincubation at 50 °C. Bovine plasma powder and egg white inhibited the formation of the new polypeptide.

Keywords: *Beef heart; myofibrillar protein; proteolysis; protease inhibitor; gelation*

INTRODUCTION

The American meat industry produces over 100 million lbs of beef hearts annually (USDA, 1997). Converting this surplus low-grade meat into surimi-like material can increase the profit margin and provide nutritious meat products for the consumers. However, one of the problems with beef cardiac muscle is its poor gelation ability compared with skeletal muscles. Gelation is one of the main physicochemical processes involved in the cooking of comminuted muscle foods for which the major contribution by myofibrillar proteins has been fully recognized (Ziegler and Foegeding, 1990). The ability of muscle proteins to form a cohesive gel matrix either in isolated protein model systems or in processed meats is determined by a variety of intrinsic and extrinsic factors, including muscle fiber type, protein structure and concentration, pH, ionic strength, and redox potential (Xiong, 1997). A number of researchers have also shown that cooking temperature and specific heating regimen critically influence the physical properties of muscle protein gels thereby affecting the textural properties of meat products (Montejano et al., 1984; Foegeding et al., 1986; Xiong and Blanchard, 1994; Lan et al., 1995).

Fish surimi is a typical muscle food in which the sensitivity of protein gelation to the heating temperature and process is fully manifested. Gelation of surimi from many fish species is highly dependent on the heating rate and the cooking temperature, exhibiting either gel-strengthening at low temperatures (known as "suwari") (Lanier, 1986) or gel-softening at an intermediate temperature range (known as "modori") (Makinodan et al., 1985). More detailed investigations have revealed the involvement of certain endogenous enzymes with both phenomena (Wasson, 1992; Morrisey et al., 1993; Joseph et al., 1994; Kołodziejska and Sikorski, 1996). Thus, by incorporating proper protease inhibitors and controlling the incubating temperature, gelation of many fish surimi can be improved (Lee, 1986).

While the heating effect and the role of endogenous proteases have been elucidated in fish surimi gels, little is known about such effects on the gelation of surimi-like materials prepared from terrestrial animals, particularly gelation of surimi made from beef heart muscle. The limited reports on gelation of land animal proteins have been inconsistent or contradictory. Young et al. (1992) provided some inconclusive evidence of proteolysis in beef cardiac myofibril preparation. Park et al. (1996) observed some enzymic proteolysis in surimi-like materials from pork muscle, while Liu and Xiong (1997) indicated that gel-weakening of myofibrillar proteins from chicken breast muscle was unlikely caused by common endogenous proteases. So far, there is no published report on the endogenous proteases as related to the gelation of beef heart surimi. Hence, the objectives of this study were to determine the possible existence of proteases in beef heart surimi, to investigate the impact of endogenous proteases on beef heart surimi gelation, and to measure the changes in surimi gelation properties after treatment with protease inhibitors.

MATERIALS AND METHODS

Materials. Fresh beef hearts (24 h postmortem) were obtained from a local meat packing plant. The hearts were individually vacuum-packaged in Cryovac polyethylene bags (Cryovac Division, W. R. Grace & Co.-Conn., Duncan, SC) and stored frozen in a −29 °C blast freezer. Fresh eggs were purchased from a local retail store and whole egg white (EW) was separated from yolk prior to use. Bovine plasma powder (BPP, AMP 600) was kindly donated by AMPC Inc. (Ames, IA). Soy protein isolate (SPI) was obtained from Protein Technologies International (St. Louis, MO). Azocasein, phenylmethanesulfonyl fluoride (PMSF), E-64, propyl gallate, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All the other reagents were at least analytical grade.

Preparation of Beef Heart Surimi (BHS). The method employed to prepare BHS was essentially the same as de-

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S0021-8561(97)01042-X CCC: $15.00 © 1998 American Chemical Society
Published on Web 07/22/1998

scribed by Wang et al. (1997). Frozen beef hearts were thawed at 2 °C overnight. After the adipose tissue, caps, and large blood vessels were trimmed off, the lean cardiac muscle was ground with a food grinder (Kitchen Aid Inc., St. Joseph, MI). Propyl gallate (0.02% of ground muscle, dissolved in 15 mL 20% ethanol) was immediately mixed with the ground muscle. To prepare BHS, the ground muscle (1000 g) was initially washed 2 times with distilled water (10 L). The mixture was stirred using a spatula for 2 min and let set for 15 min. The washed slurry was separated with two layers of nylon screen (hole size 1 mm). The paste was subsequently mixed with 5 L sodium phosphate buffer (25 mM, pH 6.0) and blended using a Waring blender at high speed for 1 min. The homogenate was adjusted to pH 6.0 and centrifuged at 3000*g* for 15 min (4 °C). The pellet was collected and pooled. Cryoprotectants (4% sucrose, 4% sorbitol, 0.2% sodium tripolyphosphate, all based on the final pellet weight) was blended with the surimi. The samples were packaged in Whirl-Pack plastic bags (10 × 20 cm) (Nasco, Fort Atkinson, WI) and stored frozen at −29 °C before use.

Gelation. Two methods were employed to assess the gelation properties of BHS. Dynamic rheological testing was done to measure the kinetic profile of the BHS samples during the heat-induced sol → gel transition. After gelation, a penetration test was run to measure the physical strengths of BHS gels.

Dynamic Rheological Testing. Storage modulus (*G*′), a measure of gel elasticity, was monitored as described by Xiong (1993). The pellet of BHS was suspended (30 mg/mL of protein) in 0.6 M NaCl, 50 mM sodium phosphate buffer (pH 6.0). The sol was set at 4 °C for 18 h to ensure maximal protein solubility prior to rheological measurements. Nondestructive, oscillatory measurements of the protein suspensions during gelation were performed by using a Bohlin VOR rheometer (Bohlin Instruments, Inc., Cranbury, NJ) equipped with parallel plates (upper plate diameter 3.0 cm). After the sample was mounted, a thin layer of silicon oil was applied along the edge to prevent moisture loss. The temperature between the plates was calibrated by using a Thermolyne digital pyrometer (Barnstead/Thermolyne Corp., Singapore, model PM20700). Four heating procedures were run to determine their effects on BHS gelation: (1) linear heating (1 °C/min) from 20 to 40 °C, incubating at 40 °C for 2 h, followed by linear heating (1 °C/min) to the final temperature of 75 °C; (2) same as above except that the BHS sample was heated to and incubated at 50 °C before linear heating to 75 °C; (3) same as above except that the BHS sample was heated to and incubated at 60 °C before linear heating to 75 °C; (4) one-step linear heating from 20 to 75 °C at 1 °C/min (control). During gelation, the samples were sheared at a fixed frequency (0.1 Hz) with a maximum strain of 0.02.

Instron Penetration Testing. BHS was prepared into a 7% protein sol in 0.6 M NaCl, 50 mM sodium phosphate buffer (pH 6.0). Aliquots of 5 g of sol were weighed into individual glass vials (15.5 mm in diameter, 65 mm in length) and covered with Parafilm and aluminum foil to prevent moisture loss. After setting for 18 h at 4 °C, the vials were incubated in water baths at the following temperatures: 30, 40, 50, 60, or 70 °C for 1, 2, and 3 h prior to final cooking at 75 °C for 25 min. As a control, another sample was heated at 75 °C for 25 min directly. After cooking, gels were cooled immediately in ice water. Gel penetration force was measured according to the method of Xiong (1993). A model 4301 Instron universal testing machine (Instron Corp., Canton, MA) equipped with a flat-end (diameter of 12.5 mm) steel rod was used to compress the gels inside the glass vials (which had been equilibrated at room temperature for 40 min) with a cross-head speed of 20 mm/min. The force required to rupture the gel (the first peak occurred during the penetration) was used to indicate gel strength.

To determine the possible involvement of endogenous proteases in BHS gel-weakening, five different protease inhibitors were incorporated into the BHS sol (Table 1) in another set of experiment. Gels were prepared by cooking sols using the following procedures: (1) slow linear heating at 0.5 °C/min from 20 to 75 °C followed by incubation at 75 °C for 25 min; (2) incubation at 50 °C for 2 h prior to final cooking at 75 °C for 25 min; (3) incubation at 60 °C for 2 h followed by final cooking at 75 °C for 25 min; and (4) isothermal cooking at 75 °C for 25 min. Gel strengths of the protease inhibitor-treated samples were measured as described above.

Table 1. Formulation of Beef Heart Surimi Gels

ingredients,[a] g	control	gel 1	gel 2	gel 3	gel 4	gel 5
surimi, g	64	64	64	54	54	54
egg white, g				10		
BPP, g					1.5	
SPI, g						1.3
PMSF, μmol		40				
E-64, μmol			0.28			
water, mL	33.5	33.5	33.5	33.5	42	42.2
NaCl, g	2.5	2.5	2.5	2.5	2.5	2.5
total, g	100	100	100	100	100	100

[a] Since EW, BPP, and SPI contained proteins, the amount of BHS was adjusted to make the beef heart surimi sol contain 7% protein. The formulation was based on Lee (1986) with some modifications.

Water-Holding Capacity (WHC). WHC was determined according to the method of Foegeding and Ramsey (1987). Cooked gels were cooled immediately in ice water and let set at 4 °C overnight. After extraneous water was blotted off, the gel was put into a thimble made with three pieces of Whatman #2 qualitative filter paper (one piece of diameter 5 mm on the outside, two pieces of diameter 7 mm on the inside) and centrifuged at 5000*g* for 15 min (4 °C). The centrifuged gels were dried in a 100 °C oven for 24 h to analyze its remaining moisture (AOAC, 1990). WHC was computed as gel weight after centrifugation minus dry weight, divided by the protein content.

Proteolytic Activity. Identification and measurement of the activity of endogenous proteases in BHS were performed as described by An et al. (1994). Crude enzyme extract (CEE) was prepared by blending BHS with sodium phosphate buffer (0.15 M sodium phosphate, 0.15 M NaCl, pH 7.0) at a 1:1 ratio in a Warring blender and centrifuged (17500*g* for 30 min, 4 °C). The supernatant was collected and used as CEE.

Preliminary experiment showed the existence of proteases. Hence, to determine the optimum pH for the proteases, the reacting media were adjusted to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0 with McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M sodium citrate). An aliquot of 1.875 mL McIlvaine's buffer was mixed with 1.125 mL azocasein solution (10 mg/mL) and 0.75 mL CEE. The mixture was incubated at 50 °C for 1 h. To determine the optimum temperature for the proteases, the reacting medium was adjusted to pH 5.0 by using McIlvaine's buffer and incubated at 30, 40, 45, 50, 55, and 60 °C for 1 h. After incubation, an aliquot of 0.6 mL of 50% trichloroacetic acid (TCA) solution was added and the mixture was centrifuged at 5000*g* for 15 min (4 °C). A 2.4-mL aliquot of supernatant was drawn and mixed with 0.18 mL of 10 N NaOH. After 3 min, the absorbance was read at 450 nm. A sample blank, with CEE added at the end of incubation, was run simultaneously. Enzyme activity was expressed as ($Abs_{sample} - Abs_{blank}$).

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was done according to Laemmli (1970) by using an SE 250 Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). The separating gel was a 4−20% gradient gel. Samples for electrophoresis were prepared by blending 2 g of gel in 18 mL of dissolving solution (5% SDS, 0.1% 2-mercaptoethanol) by using a Polytron (Brinkman Instruments, Inc., Westbury, NY). The homogenized samples were incubated at 80 °C for 1 h to completely dissolve proteins and subsequently centrifuged at 5000*g* for 15 min. After measuring the protein concentration by the Biuret method (Gornall et al., 1949), the supernatant was diluted to a 2 mg/mL protein concentration with the above dissolving solution and mixed with SDS−PAGE sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol,

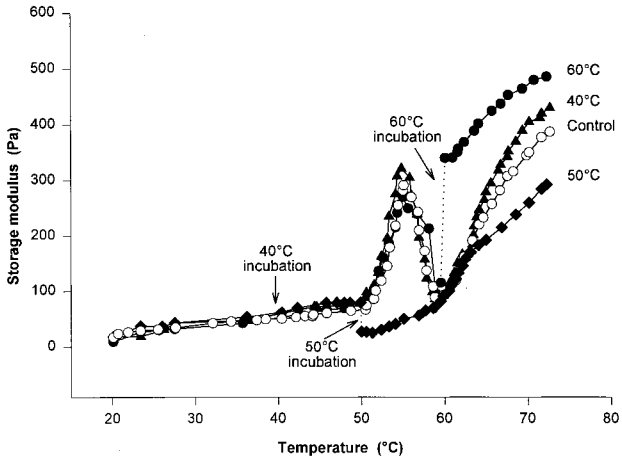

Figure 1. Changes in the storage modulus (G') of BHS (30 mg/mL protein in 0.6 M NaCl, 50 mM sodium phosphate buffer, pH 6.0) during linear heating without (control) or with preincubation at the specific temperatures indicated. The dotted lines indicate changes in G' during incubation. For exact heating procedures, see Materials and Methods.

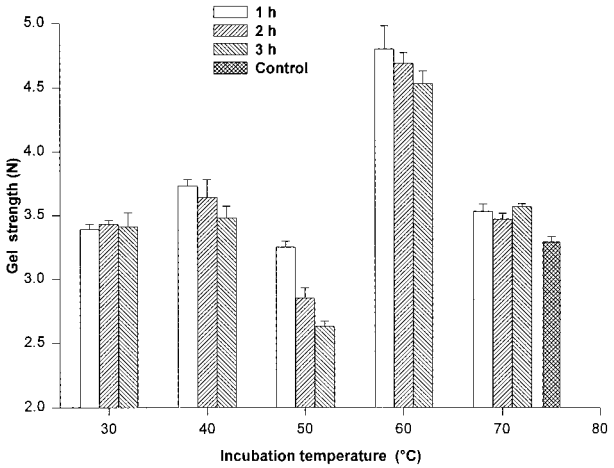

Figure 2. Effects of incubation on the strength of BHS gels (70 mg/mL protein in 0.6 M NaCl, 50 mM sodium phosphate buffer, pH 6.0). Gels were prepared by preincubating at different temperatures for 1, 2, or 3 h, followed by final cooking at 75 °C for 25 min. Control gel was prepared by cooking at 75 °C for 25 min directly.

0.125 M Tris, pH 6.8) at a 1:1 ratio. Finally, an aliquot of 20 μL from each sample was mounted to the gel.

Statistical Analysis. All experiments (except gelation with protease inhibitors) were designed as a randomized complete block. For gelation with protease inhibitors, the design was a randomized block split plot with the whole plot factor being the incubating temperatures (four levels) and the split plot factor being protease inhibitors (five kinds plus one control). The blocking factor was the replicate run on different days. Each experiment was replicated at least two times. Analysis of variance was done by using linear models of the Statistix 3.5 software package (Analytical Software, Inc., St. Paul, MN) for PC. Differences between means were compared by using LSD (Snedecor and Cochran, 1989). The significance level was set at 0.05 unless otherwise specified.

RESULTS AND DISCUSSION

Gelation. *Dynamic Rheological Testing.* The development of storage modulus (G') during the sol → gel evolution with incubation and linear heating was shown in Figure 1. Linear heating resulted in a typical rheogram of BHS sol with G' peaking at 55 °C and dipping to a valley at 61 °C. The sharp decline in G' after 55 °C was not well understood, although it has been suggested to result from denaturation of myosin tail (Egelandsdal et al., 1986). The maximum transition temperature, 55 °C, was approximately 5 °C higher than that for beef skeletal myofibrils (Xiong et al., 1995), suggesting that cardiac myosin was more heat resistant. During incubation at 40 °C, there was little change in G' and the overall rheological pattern did not deviate from the control. However, during incubation at 50 °C, the gel elasticity (G') dropped substantially. The most striking result of the 50 °C incubation was the diminishing of the transition at 55 °C during the subsequent linear heating. Correspondingly, the final G' was also much lower than for any other samples including the control ($P < 0.05$). The samples incubated at 60 °C, which had gone through the 55 °C transition from the "warm-up" linear heating, did not show any decrease in gel elasticity (G'), and the final G' value was the highest of all samples ($P < 0.05$). It was reported that prolonged incubation at 60 °C caused gel-weakening in some fish surimi such as Pacific whiting (Chang-Lee et al., 1989). This gel-softening phenomenon was attributed to myosin hydrolysis by some heat-activated proteases. These marine proteases were most active at temperatures close to 60 °C (Mackinodan et al., 1963; Wasson et al., 1992). Note that the incubation temperature that caused gel-weakening of BHS was 50 °C, which was 10 °C lower than the reported 60 °C for most of the fish surimi.

Instron Gel Penetration Testing. This test was performed on gels which had been cooked and cooled to room temperature. When the sol of BHS (7% protein) was incubated at 40 or 50 °C, there was a significant decrease in gel strength (gel penetration force) (Figure 2). In contrast, the incubation temperature of 60 °C produced gels with the highest strength. The results were consistent with those from the dynamic rheological tests (G', Figure 1). No significant changes in gel strength were noted for samples incubated at 30 and 70 °C even after 3 h preincubation. We postulated that 60 °C was the favorable temperature for BHS to form a three-dimensional gel matrix and this may be thermodynamic in nature (Wu et al., 1991). As Ferry (1948) hypothesized, protein gelation follows a two-step process: denaturation and aggregation. These two sequential reactions obviously took place in a concerted manner at 60 °C for BHS protein and, hence, resulted in an optimal gel strength.

The gel-weakening effect of the 50 °C incubation is worth particular attention. Park et al. (1996) also noticed a decrease in gel strength of pork surimi preincubated at 50 °C. They suggested that some proteases were associated with the myofibrillar protein preparation which could not be removed by extensive washing and centrifugal separation. Saeki et al. (1995) investigated the effect of different heating methods on the gel-forming ability of frozen surimi prepared from Chum salmon. They also observed a marked decrease in gel strength when the gels were incubated for a prolonged time between 40 and 60 °C. They attributed this to the proteolytic effect of cathepsin L. Young et al. (1992) speculated the existence of enzymic proteolysis in washed beef cardiac myofibrils. Since their results were inconsistent, they doubted the practical significance of the enzymic actions in gelation of comminuted products. Based on the results from the present study, we suggest that the gel-weakening of BHS caused by

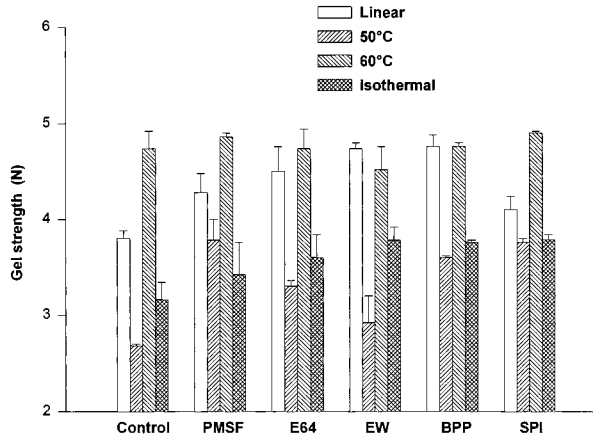

Figure 3. Effects of protease inhibitors on the strength of BHS gels (70 mg/mL protein in 2.5% NaCl, 50 mM sodium phosphate buffer, pH 6.0). Gels were prepared by (1) linear heating (0.5 °C/min from 20 to 75 °C), (2) preincubating at 50 or 60 °C for 2 h before heating at 75 °C for 25 min, or (3) isothermal heating at 75 °C for 25 min.

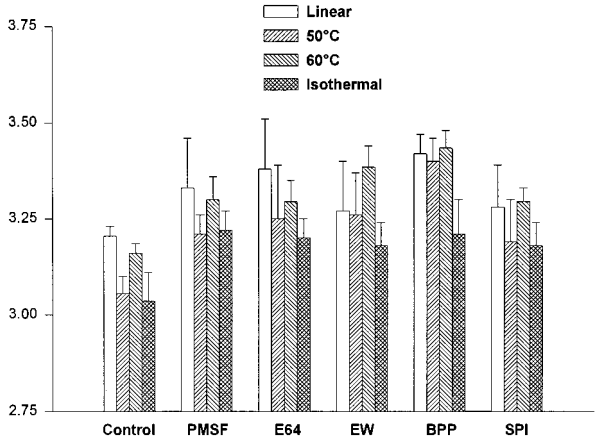

Figure 4. Effects of protease inhibitors on water-holding capacities of BHS gels (70 mg/mL protein in 2.5% NaCl, 50 mM sodium phosphate buffer, pH 6.0). Gels were prepared by (1) linear heating (0.5 °C/min from 20 to 75 °C), (2) preincubating at 50 or 60 °C for 2 h before heating at 75 °C for 25 min, or (3) isothermal heating at 75 °C for 25 min.

the 50 °C incubation resulted from proteolysis by endogenous enzymes.

To test our hypothesis, we added protease inhibitors to the BHS sol to see if the protease inhibitors had any protective effects on gel strength of BHS. We found that the effects of protease inhibitors depended on the heating procedures. As expected, the maximum protection occurred at the 50 °C incubation, especially for samples treated with PMSF, BPP, and SPI (Figure 3). In the absence of these protease inhibitors, not only did the 50 °C preincubation soften the gel, it also reduced the water-holding capacity of the gel (Figure 4).

PMSF is primarily a serine protease inhibitor, although it is also effective against cysteinyl proteases, while E-64 was specifically inhibitive against cysteinyl proteases (Bond and Butler, 1987; Carrey, 1989). Since both enzyme inhibitors showed protective effects against gel-weakening, we conclude that the active proteases in BHS consisted of cysteinyl proteases and possibly serine proteases. Although EW and BPP have been used as protease inhibitors for fish surimi commercially, their inhibitory components and mechanisms of actions were not well understood (Weerasinghe et al., 1996). Regardless, the data from this study showed that these

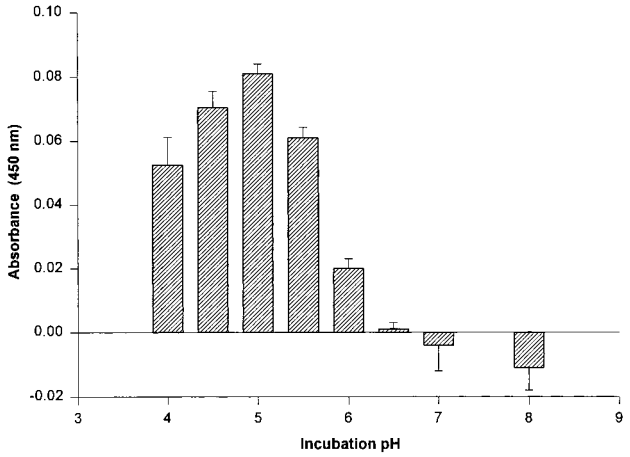

Figure 5. Activity (absorbance value) of crude enzyme extract from BHS at different pHs.

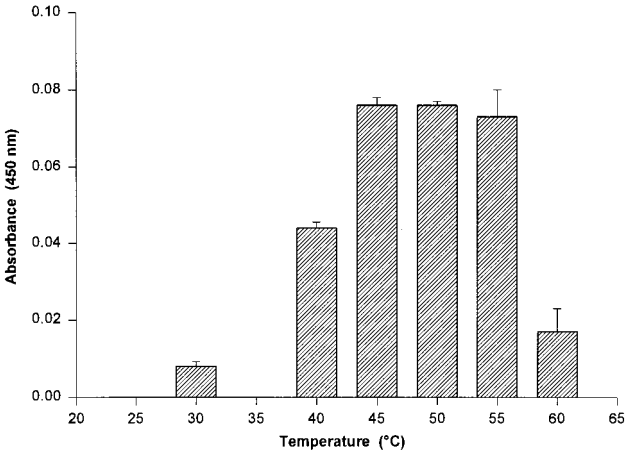

Figure 6. Activity (absorbance value) of crude enzyme extract from BHS at different temperatures.

natural ingredients had the potential for use as protease inhibitors for BHS.

Proteolytic Activity Assay. The optimum pH and temperature of the proteases were determined using azocasein as a model substrate. The optimum pHs of the enzymes were in the range 4.5−5.5 with about 25% activity remaining at pH 6.0 at which BHS gels were made (Figure 5). When the pH was above 6.5, the activity of the enzymes was diminished. Although the endogenous proteolytic enzymes in most fish surimi were reported to be most active at pH above 6.0 (Kołodziejska and Sikorski, 1996), there was compelling evidence to support that acidic cathepsins B and L were responsible for gel-weakening of some fish surimi (An et al., 1994; Jiang et al., 1996). The active proteases in BHS were most likely lysosomal proteases (cathepsins) because they were most active in the acidic pH range.

The optimum temperature of the endogenous proteases was found between 45 and 55 °C (Figure 6). This coincided very well with the decreased gel storage modulus as well as gel strength at the incubation temperature of 50 °C. Again, this optimum temperature of the proteases was lower than that reported in most fish surimi studies.

SDS−PAGE. To further verify that the gel-weakening was caused by the action of endogenous proteases, SDS−PAGE was run for BHS gels containing protease inhibitors and prepared with a 50 °C preincubation for 2 h (pH 6.0). Compared with fresh surimi sample (lane

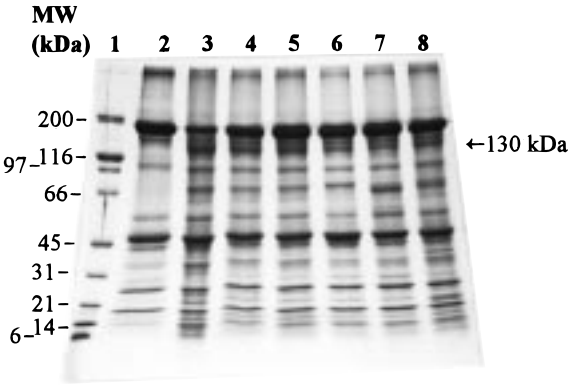

Figure 7. SDS−PAGE patterns of fresh BHS and BHS gels. Lane 1, protein standard; lane 2, fresh BHS; lanes 3−10, BHS gels with preincubation at 50 °C for 2 h without additives (lane 3) or with PMSF (lane 4), E-64 (lane 5), EW (lane 6), BPP (lane 7), and SPI (lane 8).

2, Figure 7), myosin heavy chain (MHC, 200 kDa) was partially hydrolyzed during incubation (lane 3). The resulting fragments included a polypeptide with a molecular weight around 130 kDa. With protease inhibitors, EW (lane 6) or BPP (lane 7), the 130 kDa polypeptide occurred only as a light band, indicating that the degradation of MHC was inhibited. PMSF, E-64, and SPI (lanes 4, 5, and 8, respectively) only showed slight inhibitory effects on the hydrolysis of MHC. Note that the pattern of SDS−PAGE did not correlate with gel strength for all the protease inhibitors. For instance, SPI showed significant protection against gel-weakening but it did not diminish the formation of 130-kDa polypeptide band substantially. This might be explained by the sensitivity of the two methods. Namely, even a small extent of inhibition on MHC hydrolysis could result in a significant enhancement of gel strength, which could be detected by penetration test, but this slight reduction in MHC hydrolysis might not be discerned on the SDS−PAGE gel. Another possible reason is that protease inhibitors, especially natural protease inhibitors such as BPP, EW, and SPI, might have multiple actions in inhibiting gel-weakening of surimi. Weerasinghe et al. (1996) indicated that it was difficult to correlate gel strength of surimi solely on the inhibition of proteinase. Other ingredients such as heat-coagulable proteins in BPP and EW can also increase gel strength (Porter et al., 1993). Therefore, the increased gel strength by these protease inhibitors might be due to a combined effect of proteinase inhibition and gel enhancement (Weerasinghe et al., 1996). Park et al. (1996) reported a similar proteolytic activity in beef or pork surimi (optimum temperature 50 °C, fragments 138 kDa, 128 kDa). The results of the present study strongly suggested that the endogenous proteases in BHS also played an important role in MHC hydrolysis, and their activity could be partially inhibited by protease inhibitors, such as bovine plasma powder.

In conclusion, prolonged incubation at 50 °C caused marked loss in gel strength and water-holding capacity of beef heart surimi. This most likely resulted from the action of endogenous proteases which presumably consisted mainly of lysosomal proteases (cathepsins). Hence, the ideal processing procedure for beef heart surimi would include the incorporation of protease inhibitors such as bovine plasma powder, coupled with slow heating or preincubation of the protein sol at 60 °C prior to final cooking at higher temperatures.

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Received for review December 8, 1997. Revised manuscript received June 4, 1998. Accepted June 8, 1998. This research was supported by CSRS/USDA National Research Initiative (NRI) grant, under Agreement Grant 96-35500-3324.

JF971042I