CHARACTERIZATION OF THE PROTEASES INVOLVED IN HYDROLYZING PADDLEFISH (*POLYODON SPATHULA*) MYOSIN

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ABSTRACT

An extract from paddlefish surimi possessed activities of B, L, and H-like cathepsins. The optimal pH was around 5.0 for cathepsins B and L, and was between 6.0-6.5 for the H-like cathepsin. The enzyme activities were not impaired by heating at 40C for 20 min. However, the protease extract lost about 20% of its cathepsin B, 50% B+L, and 90% H-like cathepsin activities after heating at 50C for 20 min. The activity of H-like cathepsin was not inhibited by E-64, suggesting that it did not belong to the known cysteine protease group. The protease extract was capable of hydrolyzing myosin heavy chain, producing a major fragment(s) around 140 kDa. Degradation of myosin by the protease extract was substantially reduced by protease inhibitors including E-64, a protease inhibitor mixture, and bovine plasma powder.

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INTRODUCTION

Paddlefish (*Polyodon spathula*) is one of the largest fresh water fish in North America. It can be produced in large quantity through reservoir ranching or polyculture with other species (Mims 1991; Semmens and Shelton 1986). Hence, paddlefish is a promising species for the American aquaculture industry. Traditionally, paddlefish is valued primarily for its roe and the market for paddlefish meat is still limited (Mims 1991). To increase the profitability of paddlefish production, we used paddlefish meat for surimi preparation. Our preliminary study indicated that the color and flavor of paddlefish surimi were satisfactory, but its gel strength was weakened with incubation around 40C (Lou *et al.* 2000). This gel-softening problem was similar to the well-documented modori phenomenon in other surimi such as Pacific whiting surimi (An *et al.* 1994).

It has been recognized that endogenous proteases are responsible for the softening of Pacific whiting fillet, mince, and surimi gel. However, there still exist some controversial views concerning the origin and nature of the proteases responsible for softening of Pacific whiting meat (Wasson *et al.* 1992). Early studies indicated that acidic cathepsins, particularly cathepsins A, B, C, and D, were unlikely candidates because they could not hydrolyze myofibrillar proteins above pH 7.0, the typical pH of most fish surimi (Erickson *et al.* 1983; Mackinodan *et al.* 1982). Other evidence, however, suggests that heat-stable neutral and alkaline protease(s) may be responsible for gel-weakening of whiting surimi (Busconi *et al.* 1984; Mackinodan *et al.* 1985; Su *et al.* 1981a, b). These proteases may include both serine and cysteine proteases, i.e., proteases containing serine and cysteine residues at their catalytic center (Toyohara *et al.* 1990). This information is crucial for investigating the mechanism of the proteases and for establishing effective processing procedures to control their activities. Recent studies have documented the thermal and pH profiles of cathepsins B and L, indicating that they are the likely proteases responsible for weakening of the surimi gels from Pacific whiting and mackerel (An *et al.* 1994; Jiang *et al.* 1994, 1996, 1997; Seymour *et al.* 1994). Furthermore, cathepsins B and L from these fish species are also able to hydrolyze myofibrillar proteins near pH 6.5-7.4, substantiating their ability to impair gelation of surimi (An *et al.* 1994; Benjakul *et al.* 1998; Jiang *et al.* 1996). Despite these previous studies, it is not clear whether and what enzymes, if any, are involved in the gel-weakening of paddlefish surimi. Therefore, the objective of this study was to establish the existence of the active proteases in paddlefish to enable optimal procedures to be designed for the process of paddlefish surimi.

MATERIALS AND METHODS

Materials

The paddlefish used in this study were raised in the reservoirs of Western Kentucky and harvested when their live weight reached 7-15 kg. The fillets were prepared manually, frozen at -20C, and used within 90 days. Synthetic peptides, N-CBZ-Phe-Arg-7-Amido-4-methyl coumarin, N-CBZ-Arg-Arg-Amido-4-methyl coumarin, and L-Arg-7-Amido-4-methyl coumarin, were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescein isothiocyanate casein (FITC-casein), 7-Amino-4-methyl coumarin, fluorescein isothiocyanate (FITC), phenylmethyl sulfonyl fluoride (PMSF), L-trans-epoxysuccinylleucylamide (4-guanidino) butane (E-64), pepstatin A, and an protease inhibitor mixture (Catalog # P2714) were also purchased from Sigma Chemical Co. (St. Louis, MO). Bovine plasma powder (BPP) was obtained from AMPC, Inc. (Ames, IA). All other reagents were analytical grade.

Preparation of Protease Extract

A crude protease extract was prepared according to the method of Porter *et al.* (1996). Frozen fillets were thawed at 4C and ground through a plate with 4.5 mm orifices. The ground meat was washed twice with iced tap water and once with 0.15% NaCl in the iced tap water. The slurry was dewatered using double-layered cheese cloth. The washed paddlefish meat, referred to as "paddlefish surimi", was blended with extraction buffer (20 mM sodium acetate, 1% NaCl, 0.02% NaN_3, pH 5.8, at the ratio of 2 mL buffer to 1 g meat) using a Waring blender. After setting at 4C for 1 h, the mixture was centrifuged at 10,000 × *g* for 30 min (4C). The supernatant was collected and its protein concentration was measured using the Biuret method (Gornall *et al.* 1949).

Preparation of Actomyosin

Paddlefish actomyosin was prepared following the method for myosin isolation (Margossian and Lowey 1982) with some modifications. The actomyosin was extracted using a buffer at pH 6.5 (0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4, 1 mM EDTA, 4 mM Na-pyrophosphate). The actomyosin fraction was collected after precipitation with 35% NH_4SO_4 and centrifugation at 10,000 × *g* for 15 min. The actomyosin was dialyzed (0.6 M NaCl, 50 mM sodium phosphate buffer, pH 6.5 with the buffer changed every 4 h) and diluted to a protein concentration of 20 mg/mL before being incubated with the protease extract.

Analysis of Protease Activities

Cathepsin activities were measured using synthetic peptide as substrates.

Cathepsins B and L were assayed according to Barrett and Kirschke (1981) and cathepsin H activity was tested according to Lee *et al.* (1996). N-CBZ-Arg-Arg-Amido-4-methyl coumarin was used for measuring cathepsin B activity. L-Arg-7-Amido-4-methyl coumarin was used for measuring cathepsin H activity. Since both cathepsins B and L can hydrolyze N-CBZ-Phe-Arg-7-Amido-4-methyl coumarin, they were reported as cathepsins B+L activities (Porter *et al.* 1996).

The protease extract was adjusted to the pH values of the assay buffers and diluted to a protein concentration of 12 mg/mL. An aliquot of 500 μL extract was mixed with 1.0 mL assay buffer (0.1 M sodium citrate, 0.2 M sodium phosphate, adjusted to the target pH values 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5), 40 μL 100 mM dithiothreitol, and 500 μL substrate solution (20 μM). After incubation at 40C for 20 min, 2.0 mL stopping solution (100 mM sodium monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3) were added, and the mixture was centrifuged at 3,000 × g for 10 min. Fluorescence intensity of the supernatant was measured with excitation at 370 nm and emission at 460 nm using a FluoroMax-2 spectrofluorometer (Instruments S.A. Group, Jobin Yvon/Spex Division, Edison, NJ). The instrument was calibrated to 100% using 0.5 μM 7-Amino-4-methylcoumarin. A sample blank was prepared in parallel to each test sample except that the protease extract was added after the addition of the stopping solution.

The Impact of Inhibitors on the Activities of the Proteases

To characterize the proteases, inhibitors were added and incubated with the protease extract prior to addition of the substrate (Table 1), and the activities of the proteases were assayed as described above.

TABLE 1.
PROTEASE INHIBITORS[1]

Inhibitors	Target Proteases	Final Concentration
E-64	Cysteine	8.0 μg/mL
PMSF	Serine	100 μg/mL
Pepstatin A	Aspartic	100 μg/mL
EDTA	Metallo	4 mg/mL
Cocktail	Cysteine, serine, metallo	0.5 mg/mL
BPP	--	10 mg/mL

[1]The inhibitors and their working concentrations were selected according to Barrett and Kirschke (1981), Jiang *et al.* (1996), Sigma Catalog (Sigma Chemical Co., St Louis, MO 1999)

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to verify that the protease extract had the ability to hydrolyze the major gel-forming proteins in surimi, the diluted actomyosin (20 mg/mL) was incubated with the protease extract at 40C for 2 h with assaying buffer of pHs 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5. The impact of the inhibitors on the protease extract in hydrolyzing actomyosin was evaluated by adding the protease inhibitors into the protease extract and actomyosin mixture, followed by incubation for 2 h at the gelation pH (6.5) and gel-weakening temperature (40C) for paddlefish surimi which were established previously (Lou *et al.* 2000). The profiles of polypeptides were examined using SDS-PAGE according to the method of Laemmli (1970) with the separating gel containing 10% acrylamide and the stacking gel containing 3% acrylamide.

Experimental Design and Statistical Analysis

This experiment was a randomized complete block design. The blocking factor was the replicates, i.e, the three repeated experiments run on different days. Overall F test was done using general linear models of SAS program (SAS Inc. 1990). Differences between means were analyzed using least significant difference procedure. The level of significance was defined as $P \leq 0.05$.

RESULTS AND DISCUSSION

Effects of pH on the Activities of Cathepsins B, B+L, and H

The activities of cathepsins B and B+L from the protease extract were maximal at pH 5.0-5.5 (Fig. 1). These activities declined by 50-60% at pH 6.5 wherein the myosin heavy chain of paddlefish surimi was degraded (Lou *et al.* 2000). The optimal pH for cathepsins B and B+L observed in this study was similar to those reported for other fish species, for example, pH 5.6 for chum salmon (Yamashita and Konagaya 1991a, b), 5.5-6.0 for Pacific whiting (An *et al.* 1994, 1995), and 5.0-5.5 for mackerel (Jiang *et al.* 1996; Lee *et al.* 1993). Cathepsin H activity was negligible below pH 5.0 and was maximal between pH 6.0-6.5 which was close to the reported 6.8 from human and rat livers (Barrett and Kirschke 1981). The cathepsin H activity in the protease extract of paddlefish surimi was higher than its cathepsins B and B+L activities. This was in line with the protease profile in Alaska pollock and Pacific cod (Porter *et al.* 1996). On the contrary, the cathepsin H activity in arrowtooth flounder and Pacific whiting was much lower than their cathepsins B and B+L activities (Porter *et al.* 1996).

Activity of the protease extract was also tested with FITC-casein as substrate. Surprisingly, the protease extract did not exhibit any noticeable ability to hydrolyze

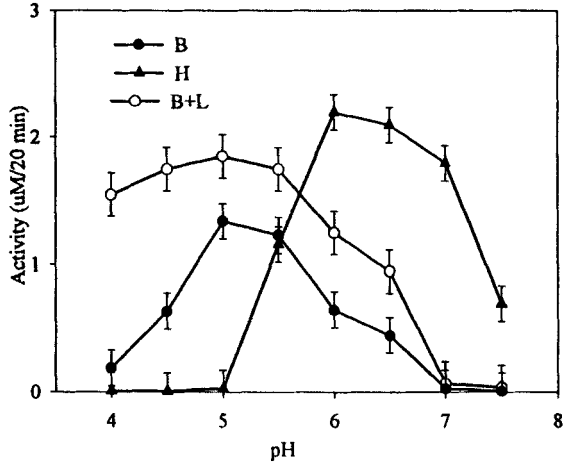

FIG. 1. THE pH PROFILE OF THE CATHEPSINS B, B+L, AND H ACTIVITY IN THE PROTEASE EXTRACT
The proteases were assayed at 40C for 10 min.

casein (data not presented). This was different from the protease extract of Pacific whiting (An *et al.* 1994) which has been shown to be highly active in hydrolyzing casein. The limited hydrolyzing ability of the protease in paddlefish meat might explain why only its myosin heavy chain was partially hydrolyzed and the resulting fragments were not degraded further (Lou *et al.* 2000). In contrast, the proteases from Pacific whiting hydrolyzed not only the myosin heavy chain but also the resulting fragments into smaller peptides (An *et al.* 1994).

Thermal Stability of the Proteases

After incubating at 40C for 20 min, none of the proteases showed any decrease in their activities (Fig. 2). However, with incubation at 50C for 20 min, the protease extracts lost a significant portion of its activities, especially its cathepsin H activity which was 90% lower than the control. It appeared that the proteases in paddlefish muscle did not belong to the class of heat-activated enzymes which hydrolyze myofibrillar proteins at elevated temperatures (Kołodziejska and Sikorski 1996). Hence, the optimal temperature of the proteases from paddlefish was different from other fish such as carp (Makinodan and Ikeda 1969), Atlantic croaker (Lin and Lanier 1980), and Pacific whiting (An *et al.* 1994) which have been shown to have optimal temperatures between 55C and 60C for their proteases.

Impact of Inhibitors on the Activities of Cathepsins B, B+L, and H

The nature of the enzymes could be inferred from their responses to inhibitors. In this study, PMSF, pepstatin A, and EDTA did not significantly inhibit the cathepsins B and B+L activities (Fig. 3), indicating that these enzymes were not

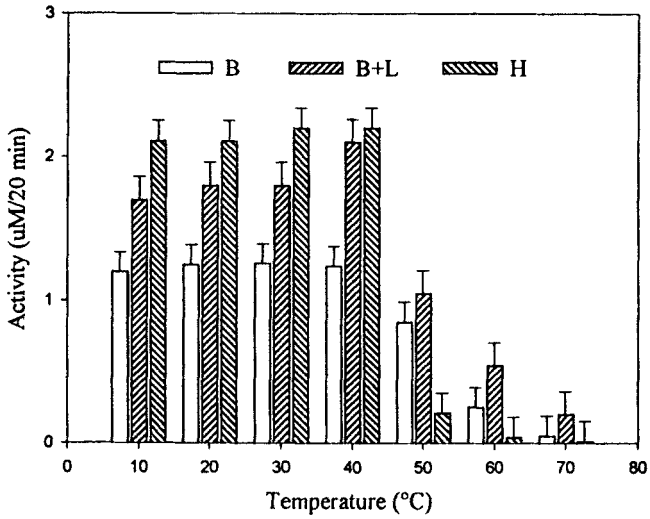

FIG. 2. THERMAL STABILITY OF PADDLEFISH PROTEASE EXTRACT
The protease extract was heated at different temperatures for 20 min.The proteases were assayed at 40C for 10 min. The cathepsin B and B+L activities were assayed at pH 5.0 and cathepsin H activity was assayed at pH 6.0.

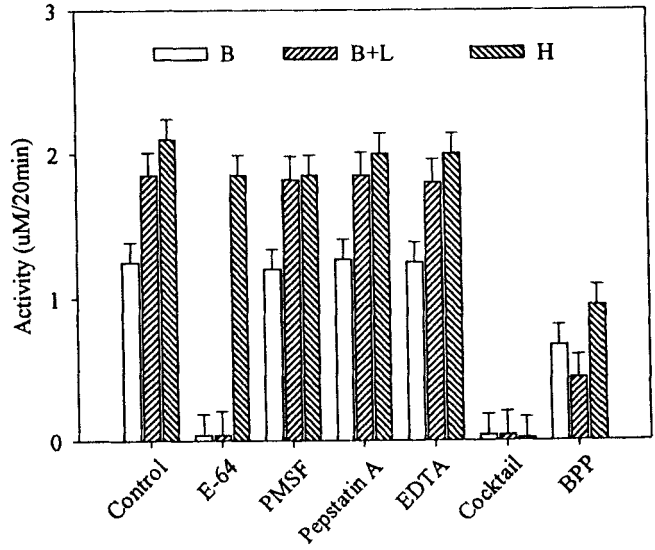

FIG. 3. EFFICACY OF THE PROTEASE INHIBITORS AGAINST PADDLEFISH PROTEASES
The proteases were assayed at 40C for 10 min. The cathepsin B and B+L activities were assayed at pH 5.0 and cathepsin H activity was assayed at pH 6.0.

serine-, aspartyl-, or metallo-proteases (Barrett and Kirschke 1981; Bond and Butler 1987). E-64 completely inhibited the cathepsins B and B+L activities, suggesting that these proteases could be cysteine proteases, which are specifically inhibited by E-64 (Barrett and Kirschke 1981; Bond and Butler 1987). This was consistent with the cathepsins B and L activities found in Pacific whiting and mackerel (An *et al.* 1995; Jiang *et al.* 1996).

The cathepsin H activity was completely suppressed by the inhibitor cocktail, but it was not affected by any individual chemical inhibitors used in this study. It seemed that cathepsin H activity in paddlefish was different from the known cysteine proteases (Barrett and Kirschke 1981). Since it was not inhibited by E-64, it was tentatively named as cathepsin H-like protease.

Bovine plasma powder was effective in reducing the activities of all the three cathepsins. In commercial practice, BPP has been used as an ingredient to enhance gelation of surimi that has serious gel-softening (modori) problems such as Pacific whiting (Lee 1986). The results from this study indicated that BPP possessed the ability to inhibit the proteases in paddlefish meat. Hence, BPP could also be used to enhance the gelation of paddlefish surimi.

Hydrolysis of Paddlefish Actomyosin by the Protease Extract

The protease extract hydrolyzed myosin heavy chain, as evidenced by the reduction of its band intensity when the incubation pH was below 6.5 (Fig. 4). Correspondingly, a new polypeptide band with molecular weight slightly higher than 140 kDa (indicated by arrow) was formed and this new band was not further degraded. Addition of E-64, a mixture of protease inhibitors, and BPP significantly reduced the hydrolysis of the myosin heavy chain, but PMSF, pepstatin A, and EDTA did not affect degradation of myosin heavy chain (Fig. 5). Presumably, all three cathepsins (B, L, and H) could have been involved in myosin degradation because they were active at the temperature and pH used for myosin hydrolysis in this study (Fig. 1, 2). In line with this speculation is that cathepsin L is the most active cathepsin in many species (Barrett and Kirschke 1981) and it can hydrolyze a variety of proteins, including connectin, nebulin, α-actinin, troponins, and actomyosin (Jiang *et al.* 1996; Yamashita and Konagaya 1991b). Hence, cathepsin L is a likely candidate for degrading paddlefish myosin. For similar reasons, cathepsin B is also a potential candidate for degrading paddlefish myosin. Based on its pH and temperature profile, the H-like cathepsin might play a significant role in degrading paddlefish myosin. Nevertheless, this needs to be confirmed by further study with purified H-like cathepsin because previous study showed that cathepsin H could not hydrolyze myofibrillar proteins (Jiang *et al.* 1997).

It seemed that the results from this study (with protease extract) corroborated with the results from paddlefish surimi which exhibited a hydrolysis of myosin heavy chain at pH 6.5 (Lou *et al.* 2000). Nevertheless, the protease extract showed

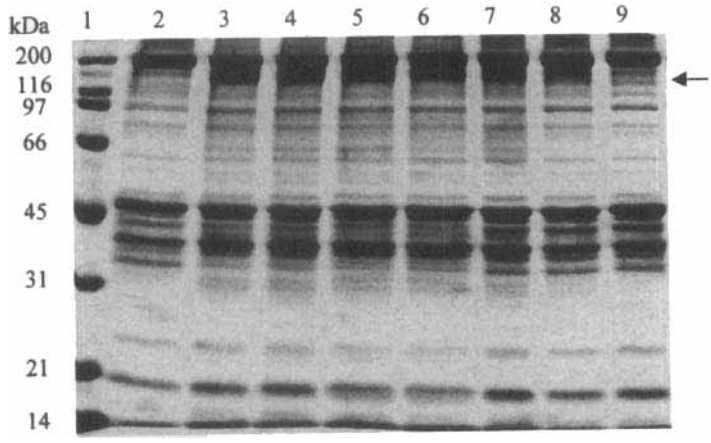

FIG. 4. EFFECT OF pH ON HYDROLYSIS OF PADDLEFISH ACTOMYOSIN BY THE PROTEASE EXTRACT FROM PADDLEFISH SURIMI

The mixtures were incubated at 40C for 2 h. Lane 1: molecular weight standard, lane 2: actomyosin, lanes 3-9: actomyosin+protease at pHs 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5, respectively. The arrow indicates a major degradation product of actomyosin.

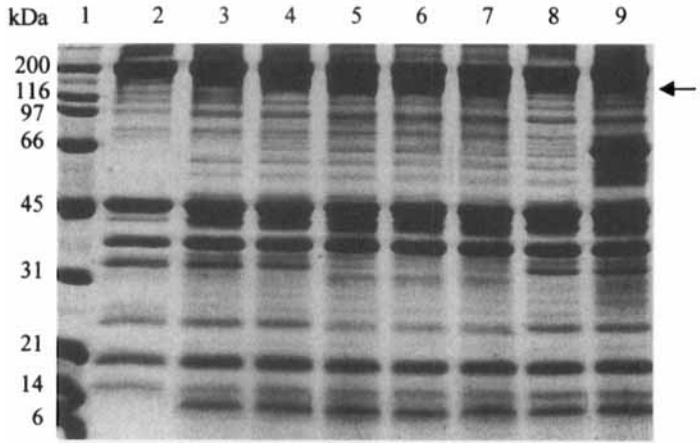

FIG. 5. EFFECT OF PROTEASE INHIBITORS ON THE HYDROLYSIS OF PADDLEFISH ACTOMYOSIN BY THE PROTEASE EXTRACT FROM PADDLEFISH SURIMI

The mixtures were incubated at 40C, pH 6.5 for 2 h. Lane 1: molecular weight standard, lane 2: actomyosin + enzyme, lanes 3-9: actomyosin+enzyme with E64, PMSF, Pepstatin A, EDTA, protease inhibitor cocktail, and BPP, respectively. The arrow indicates a major degradation product of actomyosin.

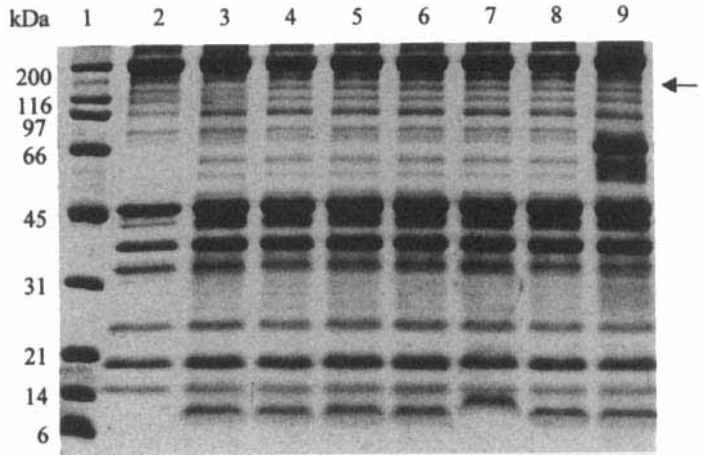

FIG. 6. THE CONTROL OF SDS-PAGE PATTERN SHOWING THAT MYOSIN HEAVY CHAIN WAS NOT HYDROLYZED (NOTE THE ABSENCE OF THE MYOSIN DEGRADATION PRODUCT IN THE PLACE INDICATED BY AN ARROW)

After the actomyosin was incubated with the assaying buffer at 40C, pH 6.5 for 2 h, it was mixed with protease extract. Lane 1: molecular weight standard, lane 2: actomyosin+enzyme, lanes 3-9: actomyosin+enzyme with E64, PMSF, Pepstatin A, EDTA, protease inhibitor cocktail, and BPP, respectively.

a lower protease activity compared with paddlefish surimi whose myosin heavy chain was almost completely degraded after 2 h incubation at 40C. The slight discrepancy may be accounted for by the fact that the enzyme in the protease extract was diluted compared to the surimi because the protease extract was prepared by using two volumes of extraction buffer (20 mM sodium acetate, 1% NaCl, 0.02% NaN3, pH 5.8). Furthermore, to reduce interfering protein bands from protease extract, only 0.2 mL protease extract was used in 3 mL hydrolyzing mixture so that the changes in myosin heavy chain could be observed more easily. Hence, it is understandable that the proportion of protease to paddlefish myosin in the hydrolyzing mixture was lower than that of the paddlefish surimi.

CONCLUSIONS

The protease extract from paddlefish surimi possessed cathepsins B, L, and H-like activities. The cathepsins B and L were likely candidates for the hydrolysis of myosin but the role of the cathepsin H-like protease in myosin hydrolysis needs to

be confirmed. Since bovine plasma powder was effective in inhibiting the degradation of myosin heavy chain, it could be used to minimize the gel-softening of paddlefish surimi.

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