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Participation of cathepsin L in modori phenomenon in carp (*Cyprinus carpio*) surimi gel

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ABSTRACT

Cathepsin L (Cat L) in carp (*Cyprinus carpio*) dorsal muscles was purified and its molecular weight determined by SDS polyacrylamide gel electrophoresis (SDS–PAGE) was 36 kDa. Its optimal temperature and pH were 50 °C and 5.5, respectively. The results of the effects of specific substrates, activators and inhibitors on the enzymatic activity showed that Cat L belongs to the family of cysteine proteinases containing thiol. Compared to the control, the gel strength of surimi with the addition of purified Cat L decreased significantly by 24.33% while that of surimi with both purified Cat L and inhibitors increased by 13.7% and 21.6%, respectively, suggesting the participation of Cat L in the modori phenomenon occurring in carp surimi. Both the SDS–PAGE electrophoretic pattern and microstructure figure revealed that Cat L could hydrolyse the main protein in carp surimi and was one of the enzymes involved in the modori phenomenon.

1. Introduction

During processing of surimi products, the occurrence of heat-induced degradation is a universal and severe problem. Myofibrillar components, especially myosin heavy chain (MHC), are essential for surimi gel formation. However, when the surimi is passing through the temperature zone of 40–60 °C to form a gel, its protein components, especially MHC, can be degraded, eventually very low gel strength causes surimi gel disintegration, which is detrimental to the quality of surimi products and reduces their commercial value. This process is called modori phenomenon (Hu, Morioka, & Itoh, 2010). Many researchers have revealed that this degradation is caused by endogenous proteases in fish muscles, especially serine- and cysteine-type proteases. Cathepsin L (Cat L, EC 3.4.22.15), as a typical cysteine protease, has a high activity to hydrolyse a broad range of proteins, such as myosin, actin, nebulin, insulin, myoglobin, glucagon, azocasein, histones, haemoglobin, and insoluble collagen (Kirschke & Barrett, 1987; Koga, Yamada, Nishimura, Kato, & Imoto, 1990; Yamashita & Konagaya, 1991).

Bleaching is very important in surimi processing as it removes most of the water-soluble proteins including those endogenous enzymes that cause gel disintegration, which hinders the gel formation of surimi. However, hydrolytic enzymes could not be removed completely. Cat L was reported to still remain in surimi after intensive bleaching and display high activity in many fish species (Hu, Morioka, & Itoh, 2008a). Cat L could hydrolyse myosin heavy chain (MHC) and caused heat-induced gel softening (modori phenomenon), resulting in the rapid decrease of gel strength and flexibility (Hu et al., 2010).

Cat L has already been purified from carp hepatopancreas (Futoshi, Hidehiro, Kenji, Kiyoshi, & Tadashi, 1997), arrowtooth flounder muscle (Wonnop, Soottawat, & Haejung, 2003), silver carp muscle (Liu, Yin, Zhang, Li, & Ma, 2006) and sea cucumber body wall (Zhu et al., 2008). Its optimal temperature and pH are 50–60 °C and 5.0–5.5, respectively, suitable for the occurrence of modori phenomenon; the enzyme was likely involved in the gel softening. However, the purification of Cat L from carp dorsal muscles and its modori mechanism have not been reported yet.

In this paper, using carp (*Cyprinus carpio*) as raw material, Cat L was purified from carp surimi through ionic exchange chromatography. Characteristics of the enzyme such as optimal temperature, optimal pH, thermal stability, effect of activators and inhibitors were analysed, to provide a theoretical basis for surimi processing. In addition, SDS–PAGE electrophoretic pattern and scanning electron microscopy were performed to clarify the effect of cathepsin L on surimi protein degradation, in order to elucidate the mechanism of modori phenomenon.

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2. Materials and methods

2.1. Sample

Cultured carp (*C. carpio*, mean body weight of 900–1200 g) was purchased alive from a local fish market in Hangzhou, China. The fish were instantly sacrificed, decapitated and decorticated to collect the dorsal muscles and then minced to make surimi. The carp surimi was immediately used or stored at −80 °C for future use.

2.2. Chemicals

Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, L-Arg-MCA, 7-amino-4-methylcoumarin (AMC), soybean trypsin inhibitor (STI), L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Leupeptin was purchased from Amresco, Inc., (Solon OH). β-Mercaptoethanol (β-Me) was purchased from Boyun Co. (Shanghai, China). Dithiothreitol (DTT), low molecular weight SDS–PAGE protein marker and broad molecular weight SDS–PAGE protein marker were purchased from Bio-Rad Co. (Hercules, CA). Cellulose DE-52 was purchased from Whatman Co. (Maidstone, UK). TSK-GEL was purchased from Tosoh (Tokyo, Japan; type G4000pWXL, column size, 7.8 mm ID × 30.0 cm L).

2.3. Crude extraction of cathepsin L from carp surimi

All stages were carried out at 4 °C. Actomyosin was prepared using the method of Hu et al. with a slight modification (Hu, Morioka, & Itoh, 2008b; Takashi & Arai, 1970). Carp surimi (75 g) was homogenised 3 times (DS-1, Specimen Model, Shanghai Co. Ltd., China) with low-ionic-strength sodium phosphate buffer (Buffer A, pH 6.8) and then centrifuged at 3000*g* for 10 min. The precipitate was collected and dissolved in three volumes of Buffer A containing 0.6 M NaCl (Buffer B, pH 7.5). This component was kept overnight at 4 °C and then centrifuged at 10,000*g* for 30 min. The supernatant was then diluted with 10 volumes (v/v) ice water. After centrifugation, the precipitate was dissolved in 50 mM sodium phosphate buffer containing 0.6 M NaCl (Buffer C, pH 7.0). This composition was called AM fraction I. Actomyosin was incubated in 50 °C water for 15 min and rapidly cooled down in an ice bath, and centrifuged at 10,000*g* for 20 min. The supernatant was called heated fraction II. After 30 min, ammonium sulphate powder was added to the composition at 80% saturation, and the solution was stirred at the same time using a magnetic stirrer (HWS-2, Hangzhou Co. Ltd., China). The pH of the solution was adjusted to 5.5 and stood for 10 min before centrifugation at 10,000*g* for 10 min. The precipitate was dissolved in 20 mM sodium phosphate buffer containing 5 mM L-cysteine (Buffer D, pH 5.5) and dialysed overnight. The supernatant was called crude enzyme fraction III. After vacuum freeze-drying, the crude enzyme was stored at −80 °C for future use.

2.4. Purification of cathepsin L

The vacuum freeze-dried crude enzyme was dissolved with Buffer D before chromatography; this solution was called concentration fraction IV.

Concentrated solution and all the buffers were filtered through 0.45-μm filter membrane before chromatography. The filtered crude enzyme was subjected to a column of cellulose DE-52 (2.6 × 25 cm) equilibrated with Buffer D. After it was washed with the equilibration buffer for elution of unabsorbed protein at the rate of 0.8–1.0 mL/min, the ionic exchange chromatography was eluted at a linear gradient of NaCl from 0 to 1 M in Buffer D. The eluate which had absorbance at 280 nm was collected for cathepsin L activity assay. High activity eluates were used as ionic exchange fraction V. Then the high activity sample was injected into the TSK-GEL column connected to a HPLC pump and a UV detector, using ultrapure water at 4 °C at a flow rate of 0.5 mL/min to elute the sample and confirm its purity.

2.5. Protein determination

The protein concentration was determined by the Biuret method (Robinson & Hodgen, 1940), using bovine serum albumin (BSA) as standard.

2.6. Assay of enzyme activity

Enzyme activity was measured according to the method of Barrett (1980), Barrett and Kirschke (1981), and Hu, Morioka, and Itoh (2008c) with a slight modification. Enzyme sample (0.9 mL) was mixed with 0.50 mL of 0.4 M sodium acetate buffer containing 4 mM ethylenediaminetetraacetic acid (EDTA) (Buffer E, pH 5.5) and 20 mM freshly made cysteine. After preheating at 25 °C for 2 min, the reaction was immediately initiated by 0.50 mL of 0.1 mM substrate solution and incubated at 25 °C for 30 min; the substrate reaction was terminated by adding 3.0 mL stop reagent (0.1 M sodium acetate buffer containing 0.1 M sodium monochloroacetate). The fluorescence intensity of the liberated 7-amino-4-methyl-coumarin (AMC) was measured with a fluorescence spectrophotometer (Varian Inc., Palo Alto, CA) at an excitation wavelength of 370 nm and emission wavelength of 460 nm. One unit was defined as 1 nmol AMC in 30 min at 25 °C.

2.7. SDS–PAGE

The carp protein was extracted by the method of Bechtel and Parrish (1983). An aliquot of 20 μL from each sample was subjected to SDS–PAGE under reducing conditions. After electrophoretic running (EPS-300 Tanon vertical electrophoresis apparatus, Shanghai, China), proteins in the gel were stained with Coomassie Brilliant Blue R-250 (Laemmli, 1970).

2.8. Effect of specific substrates on the activity of purified cathepsin L

The purified cathepsin L reacted with different synthetic substrates, namely Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and L-Arg-MCA, and the enzyme activity was detected.

2.9. Temperature and pH profile of purified cathepsin L

The optimal temperature of purified cathepsin L was determined by measuring the enzyme activity of cathepsin L in the range of 0–80 °C at pH 5.5. The thermostability was measured by incubating the enzyme at 50 °C and pH 5.5 between 0 and 40 min. The optimal pH was assayed in the range of 3.5–8.0 using Buffer E at 50 °C. Z-Phe-Arg-MCA was used as a specific substrate.

2.10. Effect of activators and inhibitors on the activity of purified cathepsin L

Purified cathepsin L was incubated with 0.2 mM E-64, 0.2 mM leupeptin, 10 mM L-Cys, 4 mM β-Me, 2 mM PMSF, 0.2 g/L STI, 4 mM EDTA and 4 mM DTT at 25 °C for 15 min. After reaction, the remaining activity of the enzyme was measured.