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Purification and characterisation of cathepsin L from the skeletal muscle of blue scad (*Decapterus maruadsi*) and comparison of its role with myofibril-bound serine proteinase in the degradation of myofibrillar proteins

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ABSTRACT

The *modori* phenomenon during surimi production is caused by endogenous proteinases, especially cathepsin L and myofibril-bound serine proteinase (MBSP). Cathepsin L from the skeletal muscle of blue scad (*Decapterus maruadsi*) was purified to homogeneity by ammonium sulphate fractionation and a series of column chromatographies and revealed a single band with molecular mass of 30 kDa on SDS–PAGE. Peptide mass fingerprinting (PMF) obtained three fragments with 48 amino acid residues, which were highly identical to cathepsin L from other fish species. Its optimal pH and temperature were 5.5 and 55 °C, respectively. Meanwhile, MBSP was purified from the skeletal muscle of blue scad, and the roles of cathepsin L and MBSP in the degradation of myofibrillar proteins were compared. The results indicated that MBSP is more effective than cathepsin L in promoting the degradation of myofibrillar proteins, especially myosin heavy chain (MHC), suggesting that MBSP plays a more significant role.

1. Introduction

Blue scad (*Decapterus maruadsi*) is a kind of marine fish belonging to the family of mackerel which is popularly harvested in all tropical and temperate seas, and its production in China reached 5,40,000 tons in 2009 (Anonymous, 2010). Fish jelly product surimi is regarded as a promising product for processing. Recently, low-valued dark-fleshed fish, such as blue scad, is becoming a potential raw material for surimi production. Nevertheless, it is necessary to pay attention to the modori phenomenon (thermal gel degradation of fish jelly products at the temperature around 55 °C), which takes place during the manufacture process of surimi for most fish species, including marine fish from the family of mackerel (*Scomber australasicus*) (Jiang, Lee, Tsao, & Lee, 1997).

It is now generally regarded that endogenous proteinases, especially cathepsins and myofibril-bound serine proteinases (MBSP) are the most important endogenous enzymes, which participate in the degradation of myofibrillar proteins, leading to the modori phenomenon of surimi (Cao, Jiang, Zhong, Zhang, & Su, 2006; Cao et al., 1999; Hu, Morioka, & Itoh, 2010; Jiang et al., 1997). Among cathepsins, cathepsin L was mostly attracted attention as it may bind to myofibrillar proteins after neutral or alkaline washing. Though the gel softening effect of cathepsin B was also observed (Jiang, Lee, & Chen, 1996; Jiang et al., 1997), it can be removed effectively during the leaching processing (An, Weerasinghe, Seymour, & Morrissey, 1994). Furthermore, cathepsin L showed highest activity at 55 °C, which was identical to the temperature of modori. So far, characterisation of cathepsin L from different species of fish has been reported in chum salmon (*Oncorhynchus keta*) (Yamashita & Konagaya, 1990), mackerel (Lee, Chen, & Jiang, 1993), arrowtooth flounder (*Atheresthes stomias*) (Visessanguan, Benjakul, & An, 2003; Visessanguan, Menino, Kim, & An, 2001), silver carp (*Hypophthalmichthys molitrix*) (Liu, Yin, Zhang, Li, & Ma, 2006), and common carp (*Cyprinus carpio*) (Tsunemoto, Osatomi, Nozaki, Hara, & Ishihara, 2004). The proteolytic effect of cathepsin L on myofibrillar proteins had also been investigated (Hu, Morioka, & Itoh, 2007; Jiang et al., 1996, 1997; Ogata, Aranishi, Hara, Osatomi, & Ishihara, 1998).

On the other hand, the presence of MBSP was also proved to be crucial to the degradation of fish myofibrillar proteins. MBSP has been purified to homogeneity from freshwater fish common carp (Osatomi, Sasai, Cao, Hara, & Ishihara, 1997), crucian carp (*Carassius auratus*) (Guo et al., 2007) and marine lizard fish (*Saurida wanieso*) (Cao, Osatomi, Hara, & Ishihara, 2000). The proteolytic effect of MBSP on myofibrillar proteins and surimi products has been studied (Cao et al., 1999, 2000). The existence of MBSP in fish muscle was notified in different species of fish. Our previous research found that

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the degradation of myofibrillar proteins in the skeletal muscle of crucian carp was effectively suppressed by soybean trypsin inhibitor (STI) (Jiang et al., 2006). More recently, we exhibited that mung bean trypsin inhibitor (MBTI) was effective in preventing the degradation of myofibrillar proteins in the skeletal muscle of blue scad (Sun et al., 2010). Because the washing process does not have effect on removing MBSP, together with its optimal temperature of 55 °C and optimal pH 7.5–8.0 (Cao et al., 2000; Sun et al., 2010), MBSP is proposed to be the most probably responsible proteinase for the modori phenomenon.

To compare the specific roles of endogenous cathepsin L and MBSP in the degradation of myofibrillar proteins, a simultaneous study of these two proteinases is necessary. Therefore, in the present study, these two proteinases were isolated respectively from the skeletal muscle of blue scad, and their specific functions in the degradation of myofibrillar proteins were compared to make certain of the major proteinase responsible for *modori* and provide theoretical reference to prevent proteolysis during surimi production.

2. Materials and methods

2.1. Materials

2.1.1. Fish

For the purification of cathepsin L and MBSP, fresh blue scad (*D. maruadsi*) (body weight about 100 g) was obtained from January to July from a market in Jimei, Xiamen, China. After decapitation and evisceration, the fish was filleted and immediately used for experiment. Live nile tilapia (*Oreochromis niloticus*) was obtained in June and fish muscle was collected for myofibril preparation as described (Sun et al., 2010).

2.1.2. Chemicals

SP-Sepharose, Q-Sepharose, Sephacryl S-200 HR and Concanavalin A-Sepharose 4B were from GE healthcare (Piscataway, USA). High S Cartridge, bovine serum albumin (BSA), benzamidine, ethylene diaminetetra acetic acid (EDTA) and protein marker for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) were products of Bio-Rad (Richmond, CA). Benzyloxycarbonyl-Phe-Arg-4-methyl-coumaryl-7-amide (Z-Phe-Arg-MCA) and other synthetic fluorogenic peptide substrates (MCA substrates) were obtained from Peptide Institute (Osaka, Japan). L-3-Carboxy-*trans*-2, 3-epoxypropionyl-L-leucine-4-guanidinobutylamide (E-64) was from Amresco (Solon, OH). Chymostatin, leupeptin, Pefabloc SC and aprotinin were products of Roche (Mannheim, Germany). Prestained protein marker for Western blot was obtained from New England BioLabs (Beverly, MA). Casein was a product of Wako chemicals (Japan). Polyclonal antibodies of rat anti-red sea bream myosin heavy chain (MHC) and rat anti-red sea bream α-actinin were gifts from professor Tachibana of the Faculty of Fisheries, Nagasaki University, Japan. Polyclonal antibodies of rat anti-carp actin and tropomyosin were prepared in our laboratory. Enhanced chemiluminescent (ECL) substrate for Western blot was from Pierce (Rockford, IL, USA). All other reagents were of analytical grade.

The buffer solutions were used in experiment as follow: Buffer A: 25 mM phosphate buffer (pH 6.5); Buffer B: 20 mM sodium acetate buffer (pH 4.5); Buffer C: 20 mM Tris–HCl pH 7.5 containing 0.5 M NaCl; Buffer D: 20 mM Tris–HCl buffer (pH 8.0). L-Cysteine (5 mM) is one of the necessary compositions of Buffer A, B and C.

2.2. Purification of cathepsin L

All procedures were performed under 4 °C, and the enzyme activity was measured using Z-Phe-Arg-MCA as substrate. Blue scad skeletal muscle (about 800 g) was minced and homogenised in 4-fold of ice-cold Buffer A. The homogenate was centrifuged at 8000*g* for 30 min. The resulting precipitate was used for the purification of myofibril-bound serine proteinase (MBSP) as described below, while the supernatant was fractionated with ammonium sulphate from 40% to 80% saturation. The precipitate thus produced by centrifugation was dissolved and dialysed against Buffer B containing 0.4 M NaCl extensively. The sample was subsequently applied to SP-Sepharose ion-exchange column (2.5 × 20 cm). After washing with dialysis buffer to the absorbance at 280 nm reached baseline, the column was eluted with a linear gradient of NaCl from 0.4 to 1 M at flow rate of 1 ml/min. The enzyme active fractions were collected and dialysed against Buffer B and loaded on prepacked column of High S Cartridge (1 ml). The sample was eluted with a linear gradient of NaCl from 0 to 1 M in Buffer B at flow rate of 0.5 ml/min. The active fractions were pooled, dialysed against Buffer C and applied to Concanavalin A-Sepharose 4B affinity column (0.5 × 4 cm) equilibrated with Buffer C. The active fractions were eluted with a linear gradient of 0–0.3 M α-methyl-D-mannoside in Buffer C at a flow rate of 0.15 ml/min, and concentrated by ultrafiltration using a membrane of YM-10 (Millipore, USA) as the final purified cathepsin L.

2.3. Purification of myofibril-bound serine proteinase (MBSP)

All purification steps were carried out at 4 °C following the method of Cao et al. (2000) with some modifications. The starting material was the precipitate from the first centrifugation step of cathepsin L purification and was resuspended in Buffer A (without L-cysteine). These processes of resuspension and centrifugation were repeated four times in order to remove sarcoplasmic proteins as completely as possible. The resulting precipitate was further homogenised in Buffer A (without L-cysteine). The homogenate was then heated in boiling water while stirring until the temperature reached 55 °C and further incubated at the same temperature for 5 min followed by cooling in ice–water immediately. After centrifugation, the supernatant (crude MBSP) was adjusted to pH 4.0 using 1 M HCl and stood for 1 h. Supernatant was collected after centrifugation and was adjusted to pH 6.0 using 1 M NaOH. Extracted MBSP was dialysed Buffer D and submitted to Q-Sepharose (2.5 × 10 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of NaCl from 0 to 0.5 M in Buffer D. The active fractions were pooled and concentrated by ultrafiltration, the sample was then applied to Sephacryl S-200 HR (2.5 × 98 cm) gel filtration column and eluted with Buffer D containing 0.2 M NaCl. The active fractions were pooled and considered as partially purified MBSP.

2.4. Assay of enzyme activity toward fluorogenic substrates

Fluorogenic substrate hydrolysing activity of cathepsin L was measured using Z-Phe-Arg-MCA as substrate according to the method as described (Barrett & Kirschke, 1981). In brief, the reaction mixture was consisted of 50 μl of enzyme solution and 900 μl of 0.1 M sodium acetate buffer (pH 5.5) containing 2 mM EDTA and 1 mM DTT. The reaction was initiated after adding 50 μl of 10 μM MCA-substrate and allowed to proceed for 10 min at 55 °C. The reaction was immediately terminated by the addition of 1.5 ml stopping agent (methyl alcohol/isopropyl alcohol/distiled water = 35:30:35, v/v). The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured by a fluorescence spectrophotometer (FP-6200, Jasco, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm. One unit of enzyme activity was defined as the amount to release 1 nmol of AMC per minute.

The activity of MBSP was routinely detected using Boc-Phe-Ser-Arg-MCA as substrate. More specifically, MBSP (50 μl) were added to 900 μl of 50 mM Tris–HCl buffer, pH 8.0. The reaction was initi-