LWT - Food Science and Technology 60 (2015) 1249–1253

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Effect of iced-storage on the activity of cathepsin L and trypsin-like protease in carp dorsal muscle

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ARTICLE INFO

Article history:
Received 20 April 2013
Received in revised form
3 June 2014
Accepted 1 September 2014
Available online 16 September 2014

Keywords:
Cathepsin L
Trypsin-like protease
Carp
Actomyosin
Gel disintegration (modori)

ABSTRACT

Carps were stored in ice for 0, 2 and 4 days. The actomyosin was extracted from the ice-stored fish and the enzymatic activity of cathepsin L and trypsin-like protease in the extract was assayed. The two enzymes remained in the carp actomyosin despite intensive leaching during the extraction procedure. The activity of cathepsin L decreased slightly after 2 days of storage and remained relative constant thereafter, suggesting the enzyme was only slightly affected by the short term iced-storage. However the activity of the trypsin-like protease increased rapidly with iced-storage time, indicating that it might have been activated during the short term storage in ice.

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1. Introduction

As a potential raw material for a wide variety of products, surimi has become increasingly popular due to its unique textural properties and high nutritional value (Benjakul, Visessanguan, Hongkaew, & Tanaka, 2005). The freshness of the raw fish material is considered to be a crucial factor in determining surimi's quality (Benjakul, Visessanguan, Riebroy, Ishizaki, & Tanaka, 2002; Shimizu, Nomura, & Nishioka, 1986). When a fish dies, proteolytic enzymes in the muscle as well as the digestive enzymes can leak into the muscle and hydrolyze the muscle proteins (Arantxa et al., 2007). Severe proteolysis of myofibrillar proteins by the endogenous proteinases in the muscle has been directly associated with poor gel quality (An, Peters, & Seymour, 1996). Among them, cathepsin and trypsin-like proteases were reported to be highly involved in the breakdown of muscle structure in several fish species (Bahuaud et al., 2010; Klomkla, Benjakul, & Kishimura, 2014; Zhou et al., 2014).

In order to preserve the high quality of fresh fish material, the normal procedure is to store the fish in ice. Usually 2–4 days of storage is necessary for commercial transportation and for temporary storage prior to surimi processing. Some fish can be stored in ice for a longer time period and still produce good quality surimi (Lin & Morrissey, 1995; MacDonald, Lelievre, & Wilson, 1990). However, degradation of the myosin heavy chain by endogenous proteolytic enzymes has been reported to occur during iced-storage of many fish species, such as Pacific whiting, bigeye snapper, sardine, true cod, thread bream and Alaska pollock (Benjakul, Seymour, Morrissey, & An, 1997; Benjakul et al., 2002; Erickson, Gordon, & Anglemier, 1983; Seki, Oogane, & Watanabe, 1980; Toyohara, Kinoshita, & Shimizu, 1990). Based on current information, two types of endogenous proteases, serine proteinase and cysteine proteinase, have been identified as factors responsible for the disintegration of surimi gels (An, Weerasinghe, Seymour, & Morrissey, 1994; Cao, Jiang, Zhong, Zhang, & Su, 2006; Cao, Osatomi, Pangkey, Hara, & Ishibashi, 1999; Jiang, Lee, Tsao, & Lee, 1997; John, Siemankowaski, Siemankowaski, Greweling, & Goll, 1982; Kinoshita, Toyohara, Shimizu, & Sakaguchi, 1991; Yanagihara, Nakaoka, Hara, & Ishihara, 1991). In these studies, the emphasis was placed upon lysosome cathepsins, such as cathepsin B, H, L, D, S, and trypsin as well as trypsin analogs. It is therefore, very important to investigate the changes in the proteolytic activity of these enzymes during short term iced-storage of fish.

In a previous study, cathepsin L was shown to have the highest activity among the four enzymes tested (cathepsin B, H, L and trypsin-like protease). The main activity was found in a peak that was clearly separated from that of actomyosin (AM) and myosin

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http://dx.doi.org/10.1016/j.lwt.2014.09.004
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(Hu, Morioka, & Itoh, 2007, 2008a). The trypsin-like protease showed no obvious AM-binding property although it is known to be a myofibrillar-binding type proteinase. The activity level of trypsin-like protease in AM seemed to vary with fish species (Hu, Morioka, & Itoh, 2008b). Therefore, the objective of this study was to investigate the effects of short term iced-storage on the activity of cathepsin L and the trypsin-like protease in carp. The results of this study on the enzyme activity would give practice guidance to the storage of material fish for surimi and surimi products in food processing field.

2. Materials and methods

2.1. Chemicals

Z-Phe-Arg-MCA and Boc-Val-Pro-Arg-MCA were purchased from the Peptide Institute Inc. (Osaka, Japan). All other chemicals were of analytical grade.

2.2. Sample preparation

Live carps (*Cyprinus carpio*) were obtained from a local fish vender (Kochi city, Japan) and were immediately sacrificed in ice. The ice boxes inside which the fish samples were buried in were put in the refrigerate keeping at 4 °C. The water formed from the melted ice was then poured out from the ice box every day. The fish meat was then cut from the fish body directly without gutting. Two experiments were performed in this study. The first experiment used a single fish while the second experiment used three individual fishes. In the first experiment, a live carp (36.5 cm length, and 878.4 g body weight) was buried in ice. After the fish die in the ice, about 20 g of the dorsal muscle between the head and the dorsal fin was removed for actomyosin extraction (0 day sample). The fish was stored again in ice. Two days later, about 20 g of the dorsal muscle between the dorsal fin and the tail was removed for actomyosin extraction (2 days sample). The fish was stored again in ice for another 2 days, and about 20 g dorsal muscle from the opposite side of the fish was removed for actomyosin extraction (4 days sample). In the second experiment, three individual carp were used. A live carp (24.5 cm and 296.2 g) was buried in ice, after its death, the fish was used for the 0 day iced-storage. A second carp (23.0 cm and 228.5 g) was stored in ice for 2 days, while a third carp (21.0 cm and 190.53 g) was stored in ice for 4 days. Actomyosin was extracted from all ice-stored fish as described below.

2.3. Actomyosin extraction

Actomyosin was extracted using the method of Hu et al. (2007). The dorsal muscle was washed 3 times with low ionic strength buffer and then treated with one cycle of dilution-precipitation. The obtained actomyosin was dispersed in 0.6 Mol/L NaCl-50 mmol/L phosphate buffer (pH 7.0, buffer C) and used for the enzymatic activity assay and for gel filtration fractionation.

2.4. Determination of protein concentration

Protein concentration was determined by the Biuret method (Robinson & Hodgen, 1940) using bovine serum albumin (Sigma–Aldrich, Shanghai, China) as standard. To prepare the Biuret reagent, 0.375 g of cupric sulfate was dissolved in 100 mL distilled water. With constant swirling, 100 mL of 7.5% (w/v) NaOH was added to the solution. The resulting solution was then diluted to 250 mL by distilled water and stored at 2–8 °C in brown bottle. The protein solutions were subjected to Biuret test with 1:1 (v/v). For the control, same volume of distilled water was added. The solutions were shaken using a vortex (Scientz Biotechology, Ningbo, China). The color of the solution was incubated at 25 °C for 1 h and the absorbance of the protein samples at 545 nm were determined using an UV spectrophotometer (Labtech Ltd, Debrecen, Hungary).

2.5. Assay of enzymatic activity

Enzymatic activity was measured by the method of Hu et al. (2007), using two specific substrates, Z-Phe-Arg-MCA and Boc-Val-Pro-Arg-MCA for cathepsin L and trypsin-like protease, respectively (Barrett & Kirschke, 1981). For Cathepsin L, the substrate stock solution (10 mmol/L Z-Phe-Arg-MCA) was diluted to 0.1 mmol/L with cold deionized water before use. The reaction mixture comprised 0.25 mL of 0.4 mol/L sodium acetate buffer (pH 5.5) containing 4 mmol/L ethylene diamine tetraacetic acid, 20 mmol/L freshly made cysteine and 0.45 mL of buffered enzyme solution. After preheating at 25 °C for 2 min, the reaction was immediately initiated by adding 0.25 mL of 0.1 mmol/L substrate solution. The reaction mixture was incubated at 25 °C for 30 min, after which the substrate hydrolytic reaction was terminated by adding 1.5 mL of stop reagent (0.1 mol/L sodium acetate buffer containing0.1 mol/L sodium monochloroacetate). For trypsin-like protease, the assay method was similar to that of cathepsin L, but the substrate was replaced by Boc-Val-Pro-Arg-MCA, and the reaction mixture for trypsin-like protease comprised of 0.8 mL 50 mmol/L phosphate buffer (pH 7.0). The fluorescence intensity of the liberated 7-amino-4-methyl-coumarin (AMC) was measured with a fluorescence spectrophotometer (Hitachi 650-10S, Tokyo, Japan) at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. One unit of activity was defined as 1 nmol AMC liberated within 30 min at 25 °C.

2.6. Sepharose 6B gel filtration of actomyosin

The AM sample solution was loaded onto an Excell SD450 column (2.6 × 40 cm) packed with Sepharose 6B (Pharmacia Fine Chemicals, New Jersey, USA) and equilibrated with buffer C. The column was eluted with buffer C at 0.5 mL/min. Fractions of every 10 mL were collected with a fraction-collector of Gilson 202 (Gilson, Paris, France). Activity of cathepsin L and trypsin-like protease was monitored in each fraction according to the method of enzymatic assay.

2.7. Statistical analysis

All experiments were done in triplicate and data were subjected to statistical analysis by Student's *t*-test.

3. Results and discussion

3.1. Activity changes of proteinase in single fish individuals

During the extraction of actomyosin, cathepsin L and the trypsin-like protease remained in the actomyosin fraction even after several washing steps. It is therefore reasonable to assume that these enzymes remain in the actomyosin and would likely cause the autolysis of the meat protein during conventional surimi industrial processing.

Fig. 1 shows the activity of cathepsin L and trypsin-like protease in actomyosin extracted from the dorsal muscle of a single carp that was stored in ice for 0, 2 and 4 days, respectively. After 2 days of storage in ice, the activity of cathepsin L in AM dropped from 3.71 to 3.44 unit/mg. When the iced-storage was extended to 4 days, the

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