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Effect of high hydrostatic pressure (HHP) on myofibril-bound serine proteinases and myofibrillar protein in silver carp (*Hypophthalmichthys molitrix*)

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ABSTRACT

Silver carp (*Hypophthalmichthys molitrix*) is a popular cultured freshwater fish in China, and becomes a potential raw material for surimi product. However, silver carp surimi exhibits considerable gel softening (*modori*) and myofibril-bound serine proteinases (MBSP) involved in the degradation of myofibrillar proteins have been assumed to be crucial to the *modori* phenomenon. High-pressure technology has demonstrated its potential application to control enzyme-related seafood texture deterioration. Herefrom, it could be further applied to the freshwater fish processing and the effect of high pressure treatments on the activity of MBSP implicated in texture deterioration in myofibrils and crude enzyme extracts was evaluated. The inactivation kinetics of MBSP pressured from 200 MPa to 500 MPa at room temperature (20 °C) were fitted first-order kinetics, The extent of enzyme inactivation was lower in silver carp myofibrils in comparison with the crude enzyme extracts from myofibrils showing a protective effect against the high pressure treatment. The effect of HHP on MBSP myofibrillar degradation shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was associated with a corresponding decrease coincided with the elevated high pressure levels in intensity of myosin heavy chain (MHC). Dynamic rheological measurements indicated that high-pressure treatment exerted considerable influence on MBSP activities as well as proteins (mainly myofibrils), resulting in structural modifications and texture changes. In this study, 300 MPa (≥10 min) was the process condition (pressure and time) that caused apparent MBSP inactivation derived from the first order kinetic model and had the efficacy in controlling silver carp texture deterioration both verified by SDS-PAGE and dynamic rheological measurements.

1. Introduction

Silver carp (*H. molitrix*) is a popular cultured freshwater fish in China, due to its fast growth rate, easy cultivation, high feed efficiency ratio as well as high nutritional value. It is the strong earthy/musty taste and odor as well as containing too much intramuscular small bones that become major causes of decreased acceptability and limited consumption (Xu, Xia, Yang, Kim, & Nie, 2010). Consquently, its commercial value as a fresh fish is greatly compromised. Among fishery processing products, surimi is much-appreciated in China and is available in different shapes and textures. Along with the decrease of marine fish resources and increasing demand of surimi products, therefore, low-valued freshwater fish, such as silver carp, become a potential raw material for the future. However, silver carp surimi exhibited considerable gel softening (*modori*) in the course of heating, resulting in a decrease in textural quality, eventually having a negative effect on acceptance and price of the surimi products (Liu, Yin, Zhang, Li, & Ma, 2006). The occurrence of *modori* always accompanies the breakdown of myosin heavy chain (MHC) because it is essential for the gelling capacity of surimi systems, and the degradation of MHC finally reduces the elasticity and the commercial value of fish jelly products (Cao et al., 1999). Many researchers have revealed that this degradation is caused by endogenous proteases in fish muscles, such as cathepsins (An, Weerasinghe, Seymour, & Morrissey, 1994) or serine proteinases (Jiang, Lee, & Chen, 1996), especially myofibril-bound serine proteinases (Cao, Osatomi, Hara, & Ishihara, 2000; Cao et al., 1999). Fish jelly product was made by myofibril protein washing out the sarcoplasmic protein of fish muscle, repeated washing using water or low alkaline water is a necessary procedure in surimi processing as it removes most of soluble lysosomal proteinases (mainly cathepsins) (Cao et al., 2004). But cathepsin L still had high residual activity due to hard to removing as a result of its close affinity with myosin, compared with the other lysosomal cathepsins (Hu et al., 2012; Li, Zhou, Zhang, Liu, & Ma, 2008). However, cathepsin L shows its preference for acidic pH (Ogata, Aranishi, Hara, Osatomi, & Ishihara, 1998) and will greatly lose its activity in the temperature range 55–60 °C. So, the involvement of cathepsin L in the degradation of myofibrillar

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proteins is actually negligible (Cao, Jiang, Zhong, Zhang, & Su, 2006). However, MBSP, which had MHC-degrading activity and consequently caused gel softening, were proved to be crucial to the *modori* phenomenon and could therefore play an important role in fish processing (Cao et al., 1999, 2004). Zhong et al. (2012) reported that MBSP were more effective than cathepsin L in promoting the degradation of myofibrillar proteins, especially myosin heavy chain (MHC), suggesting that MBSP played a more significant role.

High-pressure technology is used as an alternative to conventional thermal processing because of its advantage in a better retention of nutritional and organoleptic characteristics (Cheret, Delbarre-Ladrat, de Lamballerie-Anton, & Verrez-Bagnis, 2005). High hydrostatic pressure (HHP) processing of foods can also modify protein structure (Ko, Jao, & Hsu, 2003; Lullien-Pellerin & Balny, 2002; McArthur & Wilding, 1996; Messens, Van Camp, & Huyghebaert, 1997; Mozhaev, Heremans, Frank, Masson, & Balny, 1996) and thus enzyme activity (Ashie & Simpson, 1996; Cheret et al., 2005; Jao, Hwang, Ko, & Hsu, 2007; Lakshmanan, Patterson, & Piggott, 2005; Northrop, 2002). In food muscle, high-pressure treatment affects enzymatic activities as well as proteins (mainly myofibrillar ones), resulting in structural modifications and texture changes (Angsupanich, Edde, & Ledward, 1998; Angsupanich & Ledward, 1998; Lakshmanan et al., 2005).

HHP can either deactivate or enhance enzymatic activity in food systems, as shown by various studies on real and model food systems. A clear reduction in autolytic activity was observed at pressures above 200 MPa at elevated temperatures, and it was less affected by step-pulsed than by continuous pressurization (Hurtado, Montero, Borderías, & Solas, 2001). HPP technology has been demonstrated to inactivate a wide range of food enzymes, reducing their impact on food quality and nutritional value (Ashie & Simpson, 1996; Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998; Jao et al., 2007; Katsaros, Katapodis, & Taoukis, 2009a; Lakshmanan et al., 2005; Michels, Hei, & Clark, 1996; Riahi & Ramaswamy, 2004; Wang, Zhou, & Chen, 2008), HHP mechanism for enzyme denaturation is governed by the Le Chatelier principle, which predicts that application of pressure shifts an equilibrium to the state that occupies the smallest volume, so any reaction accompanied by volume decrease, is accelerated by elevated pressures (Cano, Hernandez, & De Ancos, 1997). However, the effects of HHP on enzyme stabilization and activation have been documented relatively little, most current applications of HHP are for inactivating various deleterious enzymes, the application of HHP to enhance enzymatic activity has been explored less extensively (Eisenmenger & Reyes-De-Corcuera, 2009a, 2009b; Mozhaev, Lange, Kudryashova, & Balny, 1996; Northrop, 2002).

It was previously reported that endogenous proteases did not seem to produce off-flavors/off-odors, their impact on textural quality which determined consumer acceptance and hence the marketability of such products. HPP was demonstrated its potential application to control enzyme-related seafood texture deterioration (Ashie & Simpson, 1996). Considering the effects of high-pressure processing on the activity of texture-related enzymes, the present study was undertaken to apply high hydrostatic pressure to reduce proteolytic activity in myofibrillar protein and to determine the efficacy of hydrostatic pressure as a tool for controlling silver fish texture deterioration. There is a relative paucity of information and research on the application of this technology in the influence on deteriorative changes directly caused by MBSP.

2. Materials and Methods

2.1. Materials and Chemicals

Silver carp (*H. molitrix*) used in this study was purchased alive from a local fish market in Wuxi, China. After decapitation and evisceration, the fish was filleted by hand and washed and the fillets were immediately used for myofibril preparation or kept at a −80 °C freezer for further use.

Boc-Phe-Ser-Arg-MCA, 7-amino-4-methylcoumarin (AMC), Diisopropyl fluorophosphate (DFP), and L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) were purchased from Sigma Chemical Co. (St. Louis, MO). 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). All other chemicals used were of analytical grade.

2.2. Preparation of Myofibrils

Myofibrils was prepared by following the method of Xiong et al. (2009) with slight modifications. One kilogram of aforementioned fish fillets were added with 10-fold of volume of ice-cold 50 mM phosphate buffer (pH 7.5), and then homogenized. The homogenate was centrifuged at 10,000 *g* using an Avanti J-25 Beckman centrifuge (Beckman Coulter, Inc., New York, NY, USA) for 30 min. After the supernatant was decanted, the precipitate was washed twice, subsequently homogenized with six volumes of chilled 50 mM phosphate buffer containing 0.6 M NaCl, pH8.0. The homogenate was filtered through a double layer of cotton gauze to remove fibrous matter. After standing for 30 min, the homogenate was centrifuged at 10000 g through a laboratory centrifuge (Sigma Laborzentrifugen, Model 4 K15, Osterode, Germany) for 30 min. The obtained supernatant was myofibrillar proteins solution for use in this study. All steps were carried out at 0–4 °C, protein concentration was determined by the biuret method (Gornall, Bardawill, & David, 1949) with bovine serum albumin as a standard.

2.3. Extraction of MBSP from myofibrils and enzymatic activity assay

MBSP were extracted according to the method of Cao et al. (2006) with some modifications from pressure-treated and untreated silver carp myofibrils, respectively. Briefly, myofibrils were homogenized with 4 volumes of 20 mM Tris–HCl buffer, pH 8.0. The homogenate was immediately heated in boiling water while stirring until the temperature reached 55 °C and further incubated at the same temperature in a water bath for 5 min, followed by immediate cooling in ice water to 4 °C. After centrifugation, the supernatant was collected and referred to as crude MBSP.

MBSP activity was determined using Boc-Phe-Ser-Arg-MCA as substrate. The incubation mixture, containing 8.5 ml of 50 mM Tris–HCl buffer (pH 8.0), 1 ml of 10 mM substrate solution and 0.5 ml of the MBSP solution in a total of 10 mL, was incubated at 55 °C for 20 min. The reaction was stopped by adding 1.5 ml of the stopping agent (methyl alcohol: n-butyl alcohol: distilled water = 35:30:35, v/v). A control test was prepared in parallel for each test sample, but the stopping agent was added before the addition of the enzyme solution. MBSP enzymatic activity was detected by measuring the fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm using a fluorescence spectrophotometer (F-7000, Hitachi, Japan). One unit of the enzyme activity was defined as the amount of activity that released 1 nmol of AMC per min.

2.4. High hydrostatic pressure (HHP) treatment

High hydrostatic pressure (HHP) processing was carried out using a FOOD-LAB 900 Plunger Press system with a maximum operating pressure of 900 MPa (Stansted Fluid Power Ltd, Stansted, UK), consisting of an HHP unit with a pressure intensifier, a single usable HHP vessel of 10 mL volume. The HP vessel was surrounded by a water circulating jacket connected to a temperature control system. Throughout the pressure treatment, the vessel temperature was controlled at 20 °C. The pressure transmission fluid used was 30% Glycerol solution. The experiments consisted of two sets of samples; silver carp myofibrils