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Endogenous proteolytic enzymes – A study of their impact on cod (*Gadus morhua*) muscle proteins and textural properties in a fermented product

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ABSTRACT

The aim of this study was to investigate endogenous proteolytic activities in a cod product and their impact on muscle proteins and textural properties during fermentation and storage. The result of specific proteolytic activities showed that cathepsins, especially cathepsin B, had the highest activities during fermentation and storage. SDS–PAGE indicated more degradation of myofibrillar proteins by cathepsin L than other proteases and that the hydrolysis by cathepsins was pronounced in the last stage of fermentation. Texture analysis showed that cathepsins had a negative impact on gel strength and this impact increased in the last stage of fermentation. However the product still had a firm texture. During storage (4 °C) for one week, no significant changes were seen in the gel strength. In conclusion, cathepsins had more impact on muscle proteins and textural properties than other proteases during fermentation but had little impact on gel strength during storage at 4 °C.

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1. Introduction

Fish has an important place in human diet because it is a good source of high-quality protein and lipids in addition to many vitamins and minerals (Fellows & Hampton, 1992). In general, fish has less cholesterol and more unsaturated fat than meat. It is a healthy food and fish consumption is increasing. However, fish is highly perishable and there is therefore a need to develop new and improved processing and preservation methods. The use of lactic acid fermentation to both preserve the fish and modify their sensory and functional properties is receiving considerable attention (Adams, Cooke, & Twiddy, 1987; Hu, Xia, & Ge, 2008; Riebroy, Benjakul, & Visessanguan, 2008; Xu, Xia, Yang, & Nie, 2010; Yin, Pan, & Jiang, 2002). Fermentation of fish has been shown to be a good method for fish processing and preservation for four reasons: (1) The rapid decline of pH during fermentation and the low pH of product inhibit the growth of most hazardous food microorganisms thus leading to an increased shelf-life (Xu et al., 2010; Yin et al., 2002). (2) The nutritional value of the fish, measured as amino acid retention, is retained (Amano, 1962). In addition, the lactobacillus has beneficial effects on gastrointestinal health (de Vries, Vaughan, Kleerebezem, & de Vos, 2006). (3) Lipolysis and protein hydrolysis by endogenous and microbial enzymes lead to formation of a unique flavour during fermentation (Casaburi et al., 2007; Zeng, Xia, Jiang, & Guan, 2013). This unique flavour could mask the musty odour and taste of some fish (Zeng, Xia, Jiang, & Yang, 2012). (4) Elastic and firm fish products are produced by fermentation. This is probably because the rapid decline in pH leads to acid-induced gelation (Xu et al., 2010).

The application of fermentation in fish processing and preservation has a long history in some developing countries of Asia and sub Saharan Africa. However, most fermentation is still conducted as spontaneous processes at household or small-scale levels and the quality of the products is highly variable (Asiedu & Sanni, 2002; Lee, 1990; Twiddy, Cross, & Cooke, 1987). There is therefore a need for a better understanding of the processes taking place during fish fermentation and how these processes can be controlled. In our previous work, physical and chemical properties of a fermented fish sausage, the mechanism of acid-induced surimi gel, and biogenic amines and microbial community in a fermented fish product were studied (Xu et al., 2010; Zeng, Xia, Yang, & Jiang, 2013; Zeng et al., 2012). However, the change in endogenous proteolytic activities and their influence on fish proteins were not investigated.

Textural characteristics that are developed during surimi processing are determinant for its quality and price. A firm and elastic texture is expected (An, Peters, & Seymour, 1996). Endogenous

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proteolytic enzymes could have influence on fish muscle proteins thus leading to a change in texture. Therefore, this impact needs to be determined. There are four groups of endogenous proteolytic enzymes that are thought to be the main contributors to soften fish: cathepsins, serine proteases, collagenases and calpains (Hultmann & Rustad, 2002, 2004; Osatomi, Sasai, Cao, Hara, & Ishihara, 1997). Cathepsin B (EC 3.4.22.1) and Cathepsin L (EC 3.4.22.15) are acid cysteine lysosomal proteases and can be activated by thiol compounds. Cathepsins play a role in protein catabolism of live fish and autolyze fish muscle during postmortem storage (Hultmann & Rustad, 2004). Serine proteases (EC 3.4.21) are heat-stable alkaline proteases. They can be classified as sarcoplasmic serine proteases (SSP) and myofibril-bound serine proteases (MBSP), and play an important role in modori degradation in heated fish (Kinoshita, Toyohara, & Shimizu, 1990; Osatomi et al., 1997). It was shown that MBSP had a great impact on skeletal muscle from silver carp when fish was made into surimi (Cao, Wu, Hara, Weng, & Su, 2005). Collagenases (EC 3.4.24.7) are metalloproteinases found in skeletal muscle. The collagen triple helix is attacked by these specific proteases (collagenases) before they are hydrolysed by other non-specific proteases (Kristjánsson, Gudmundsdóttir, Fox, & Bjarnason, 1995). Calpains (EC 3.4.22.17) are neutral Ca^{2+} activated cysteine endopeptidases in sarcoplasm of muscles. Calpains cleave proteins at specific sites and result in limited proteolysis. The large peptides produced after hydrolysis by calpains have increased the susceptibility to other proteases (Gaarder, Bahuaud, Veiseth-Kent, Mørkøre, & Thomassen, 2012; Hultmann & Rustad, 2004).

The objective of this study was to investigate endogenous proteolytic activities during fermentation and storage, and determine the respective influence of each type of enzyme on fish muscle proteins and gel strength.

2. Materials and methods

2.1. Materials

2.1.1. Fish

Cod (*Gadus morhua*) fillets were bought in a local fish market. The fish dorsal muscle fillets had been stored on ice after death for 2–3 days. They were used for experiment on the day of purchase.

2.1.2. Chemicals

Bovine serum albumin (BSA) and 1-trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorogenic substrates *N*-succinyl-Leu-Tyr-AMC (5 mg) for calpains, *Z*-Phe-Arg-AMC (25 mg) for cathepsin L, *Z*-Arg-Arg-AMC (25 mg) for cathepsin B were bought from Sigma Chemical Co. (St. Louis, MO, USA). Fluorogenic substrates Boc-Phe-Ser-Arg-AMC (5.3 mg) for MBSP, succinyl-Gly-Pro-Leu-Gly-Pro-AMC (5.3 mg) for collagenases were bought from Peptide (Japan). Molecular weight marker for SDS–PAGE was a product of GE Healthcare (Buckinghamshire, UK). High molecular weight (HMW) markers include myosin heavy chain (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa). Low molecular weight (LMW) markers include phophorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). PhastGel™ Gradient 4–15 and SDS Buffer strips were bought from GE Healthcare (Sweden). All the other chemical reagents were of analytical grade.

2.2. Preparation of fermented fish mince

Lactobacillus bacteria (LAB) was obtained from Zeng et al. (2013) in our lab and characterised in the State Key Laboratory of Jiangnan University. It was separately subcultured twice in MRS broth at 30 °C for 2 days before use.

Sample was prepared by blending 200 g cod, 2 ml bacteria suspension (the final level of cells was 6–7 log CFU/g fish mince), 4 g sodium chloride, and 6 g glucose in a small food chopper. Then seven aliquots of this mixture were put into seven beakers and incubated at 30 °C. One beaker was taken after 0, 15, 24, 39, and 48 h, respectively, for the preparation of crude enzyme extracts. After fermentation for 48 h, the rest two of the beakers were stored in a cold room (4 °C). One product was taken out after 1 day and 7 days, respectively, to make crude enzyme extracts.

2.3. Preparation of crude enzyme extracts of cathepsin B, cathepsin L, calpain, and collagenase

The samples (10 g) which were withdrawn during fermentation and storage were homogenised (Ultra Turrax homogeniser, IKA Labortechnik, Germany) in 20 ml distiled water at 12,000 rpm for 30 s. The mixture was left in a cold room (4 °C) for 30 min and stirred occasionally. Then it was centrifuged at 14,600g, 4 °C for 20 min. The supernatant was used as crude enzyme extract for proteolytic activities assay of cathepsin B, cathepsin L, calpains, and collagenases. The extracts were frozen and stored at −40 °C until analysis.

2.4. Preparation of crude extracts of MBSP

The MBSP was extracted according to the method described previously by Cao, Jiang, Zhong, Zhang, and Su (2006) with slight modification in the pH of buffer. Briefly, each 5-gram sample during fermentation and storage was homogenised in 4 volumes of 20 mM Tris–HCl (pH 7.5) at 12,000 rpm for 30 s. The homogenate was heated in boiling water to get a temperature of 55 °C and further incubated at the same temperature for 5 min before it was put on ice. After centrifugation (14,600*g*, 4 °C, 20 min) and filtration through glass wool, the supernatant was used as crude MBSP. The crude MBSP extracts were frozen and stored at −40 °C until analysis.

2.5. Determination of specific proteolytic activities

The activities of cathepsin B, cathepsin L, collagenase, calpain and MBSP were determined as previously described (Hultmann & Rustad, 2004). Briefly, appropriate assay buffer (100 μl) (Buffer A for cathepsins: 150 mM bis-Tris, 30 mM EDTA, 6 mM DTT, pH 6.0; Buffer B for collagenases and calpains: 150 mM bis-Tris, 7.5 mM $CaCl_2$, pH 6.0; Buffer C for MBSP: 150 mM bis-Tris, pH 8.0) was mixed with enzyme extract (100 μl) and the mixture was incubated at 30 °C for 10 min. The reaction was started with the addition of 100 μl 0.09 mM substrate (substrate was dissolved in distiled water). After 15 min of incubation at 30 °C, the reaction was stopped by adding 3.0 ml stopping solution (1% SDS 50 mM bis-Tris buffer, pH 7.0). The solutions were immediately put on ice. Fluorescence of AMC was measured after 10 min at 360 nm (10 nm slits) excitation wavelength and 460 nm (10 nm slits) emission wavelength (Fluorescence Spectrometer 3000, Perkin Elmer, UK). The analyses were run in triplicate. The blank was prepared using 100 μl corresponding buffer instead of enzyme extract. Activities were expressed as the increase in fluorescence and given in arbitrary units (U) based on the mean of three measurements.

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