Food Chemistry 111 (2008) 879–886

Contents lists available at ScienceDirect

Food Chemistry

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

Purification and characterisation of cathepsin L2 from dorsal muscle of silver carp (*Hypophthalmichthys molitrix*)

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ARTICLE INFO

Article history:
Received 5 October 2007
Received in revised form 22 February 2008
Accepted 29 April 2008

Keywords:
Cathepsin L2
Silver carp (*Hypophthalmichthys molitrix*)
Purification
Characterisation
Proteolytic

ABSTRACT

Cathepsin L2 was purified to homogeneity from silver carp muscle using an array of chromatography methods. The enzyme showed affinity to con A-sepharose. Although it appeared to be 78 kDa on non-reducing SDS–PAGE and gel–substrate-activity SDS–PAGE, it completely degraded into 31 kDa and 26 kDa sub-units, as well as some small polypeptides on reducing SDS–PAGE. The optimum pH and temperature of cathepsin L2 for hydrolysis of Z-Phe-Arg-MCA were pH 4.5–5.5 and 45 °C, respectively. It was stable at pH 5.5 and below 40 °C, but almost inactivated at pH 7.0 and 60 °C. Substrate specificity analysis indicated that it could hydrolyse Z-Phe-Arg-MCA but not Z-Arg-Arg-MCA or L-Arg-MCA. Cathepsin L2 was efficiently activated by Cys, DTT and β-ME, but was completely inhibited by E-64. $P_2O_7^{4-}$ and Cl^- have inhibitory effects on its activity. Cathepsin L2 showed a high K_m value of 9.5 μmol/l, but extremely low K_{cat} and K_{cat}/K_m values of 0.8 s^{-1} and 84.2 s^{-1} mM^{-1}, respectively. Except for under optimum conditions (pH 5.0, 35 °C), silver carp cathepsin L2 could also hydrolyse myosin heavy chain at softening temperatures ranging from 50 to 60 °C and at surimi pH of 6.5.

1. Introduction

Silver carp (*Hypophthalmichthys molitrix*) is reported to be the most abundant fresh water fishery resource in China with annual harvests of 4.9 million metric tons (Agricultural Bureau of China, 2002). The surimi products processed from white muscle of silver carp have a vast range of commercial prospects. However, silver carp surimi exhibits considerable gel softening (modori) in the course of heating, especially at 50 °C (Luo, Kuwahara, Kaneniwa, Murata, & Yokoyama, 2001), which leads to a poor textural quality and consequently limits the further utilisation of this enormous fishery resource.

Cathepsin L (EC3.4.22.15), a lysosomal cysteine proteinase, has been shown to be particularly powerful in degrading myofibrillar components in post-mortem autolysis (Aoki & Ueno, 1997). In recent years, the endogenous heat-stable cathepsin L in fish muscle has also been revealed to be implicated in the modori phenomenon of several fish species, especially at 50–60 °C (Ho, Chen, & Jiang, 2000; Hu, Morioka, & Itoh, 2007; Ogata, Aranishi, Hara, Osatomi, & Ishihara, 1998; Visessanguan, Menino, Kim, & An, 2001). In our previous work, it was also found that the cathepsin L activity was hardly removed, and it showed the highest residual ratio of 25.61% in the washed silver carp surimi when compared with cathepsin B and H activities (Li, Zhang, Liu, & Ma, 2004). Thus, it was suggested that the residual cathepsin L activity had the possibility to degrade surimi protein during the manufacturing of silver carp surimi products. Therefore, it is important to elucidate the relationship between the cathepsin L activity and silver carp modori for developing the processing technologies further and improving the textural quality of silver carp surimi product.

Therefore, we purified the cathepsin L from dorsal muscle of silver carp. During the purification, two active peaks with Z-Phe-Phe-MCA hydrolysis activity were isolated after cation-exchange chromatography on a SP-sepharose FF column, and these were named as cathepsin L1 and L2, respectively, according to their eluted sequence (Liu, Yin, Zhang, Li, & Ma, 2006). Cathepsin L1 has been characterised as an unglycosylated cysteine proteinase of 30 kDa and it could markedly hydrolyse surimi protein mainly at 65 °C and destroy the network structure of silver carp surimi gel (Liu et al., 2006).

Here, the apparent cathepsin L2 activity was considered to be probably an isoenzymic form of cathepsin L1, since multiple isoforms of cathepsin L have been detected in ordinary muscle of some fishes, especially some with higher molecular weight such as those in mackerel (58 kDa; Aoki, Nakano, & Ueno, 1997; Lee, Chen, & Jiang, 1993), salmon muscle (50 kDa; Yamashita & Konagaya, 1990) and pacific whiting (52.4 kDa; Benjakul, Seymour, Morrissey, & An, 1996; Seymour, Morrissey, Peters, & An, 1994). It was also shown that the cathepsin L-like of 58 kDa could

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doi:10.1016/j.foodchem.2008.04.072

hydrolyse myosin, the major textural protein in mackerel surimi at pH 6.0 (Jiang, Lee, & Chen, 1996) and had been involved in the mackerel surimi gel softening at pH 5.5–7.0 and at 40–55 °C (Ho et al., 2000). Additionally, the active isoform of pacific whiting cathepsin L of 54.2 kDa was tested to be a complex, consisting of two forms of cathepsin L of 37 kDa or 30 kDa and the 15 kDa endogenous cysteine inhibitor (Benjakul et al., 1996). A cathepsin B-like of 60 kDa from mackerel was identified as a precursor of cathepsin B that similarly to cathepsin L, also belonged to the family of papain-like cysteine proteases (Aoki, Yokono, & Ueno, 2002). Otherwise, the amino acid sequence of a precursor deduced from cDNA cloned of carp cathepsin L suggested a higher calculated molecular weight (about 43 kDa) than the purified cathepsin L of 28 kDa (Tsunemoto, Osatomi, Nozaki, Hara, & Ishihara, 2004).

Actually, in animals, except for the mature form of lower molecular weight (Ishidoh et al., 1998), cathepsins could still exist in the form of precursors (Mason, Gal, & Gottesman, 1987) and enzyme–endogenous inhibitor complexes (An, Peters, Seymour, & Morrissey, 1995). Proper acidification could help disassociation of the inhibitors, cystatin or α-cysteine, from the loose combination of the complex (Godiksen & Nielsen, 2007) and accelerate the conversion of the precursor to a mature form both with an increase in activity (McDonald & Emerick, 1995).

Silver carp cathepsin L1 had been presumed to be in an active mature form (Liu et al., 2006). However, the properties of cathepsin L2 from silver carp muscle and whether it also had been involved in the gel softening were still unclear. In this study, the further purification and some biochemical characteristics of cathepsin L2 were investigated. Moreover, its proteolytic actions on the main components of silver carp surimi protein–myosin were examined. Also, the possible form of cathepsin L2 and the difference from cathepsin L1 were discussed.

2. Materials and methods

2.1. Materials and chemicals

Live silver carp *H. molitrix* (900–1200 g per fish) was sacrificed instantly after purchasing. Then, the dorsal muscle was manually carved away from bones and filleted at 4 °C. After that, it was immediately frozen in liquid nitrogen and stored at −80 °C for further investigation.

DEAE sephacel, sephacryl S-100, SP-sepharose fast flow, con A-sepharose, media for chromatography were purchased from Amersham Biosciences (Uppsala, Sweden). Benzyloxycarbonyl-L-phenylalany-L-arginyl-4-methyl-7-coumarylamide (Z-Phe-Arg-MCA), Benzyloxycarbonyl-arginylarginine-4-methyl-7-coumarylamide (Z-Arg-Arg-MCA), L-arginyl-4-methyl-7-coumarylamid (L-Arg-MCA), 7-amino-4-methylcoumarin (AMC), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), β-mercaptoethanol (β-ME), L-cysteine (L-Cys), DL-dithiothreitol (DTT), sodium dodecyl sulphate (SDS), SDS-7 molecular weight marker (14–66 kDa), Sigma Marker (6–205 kDa), α-methyl-D-mannoside and phenylmethanesulphonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) and ethyleneglycol bis (2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Amresco Inc. (Ohio, Solon). All other chemicals used were of analytical grade.

2.2. Assay of enzyme activity and protein concentration

Enzyme activity assays for cathepsin L2 with fluorescent synthetic peptide substrate Z-Phe-Arg-MCA was essentially carried out according to Barrett and Kirschke (Barrett & Kirschke, 1981) with a few modifications. The reaction temperature was 40 °C and the pre-warming time of enzyme solution was for 2 min. The intensity of fluorescence was measured in a spectrofluorometer (RF-5300 PC) with excitation and emission wavelength of 380 nm and 460 nm. One unit of enzyme activity was defined as the amount of activity that released 1 nmol of AMC per min at the assay condition.

Cathepsin B and cathepsin H activities were routinely assayed with Z-Arg-Arg-MCA and L-Arg-MCA respectively by the method from Barrett (1980).

The protein concentration was determined as described by Lowry, Rosebrough, Farr, and Randall (1951) with BSA as the standard.

2.3. Purification

The purification procedure for cathepsin L, including crude extracting, acidification treatment, $(NH4)_2SO_4$ fractionation, ultrafiltration, ordinal chromatography on DEAE-sephacel, sephacryl S-100, SP-sepharose FF, was carried out as described by Liu et al. (2006). The second active peak corresponding to 43–56 ms/cm eluted from SP-sepharose FF was collected, concentrated and dialysed against 20 mmol/l phosphate buffer containing 5 mmol/l L-cysteine, 0.2 mol/l NaCl, 1 mmol/l $CaCl_2$ and 1 mmol/l $MnCl_2$ (pH 6.0). Then, the dialysis sample was purified by a con A-sepharose affinity chromatography (1.0 × 8 cm) equilibrated with the same buffer. The flow rate was 0.15 ml/min. After washing of the unabsorbed protein with equilibration buffer, the affinity column was eluted at a linear gradient of α-methyl-D-mannoside from 0 to 1 mol/l in equilibration buffer. The resulting samples of purified enzyme were combined, concentrated and stored at −80 °C for the subsequent characterisation.

2.4. Active-site titration by E-64

Active-site titration of cathepsin L by E-64 was essentially performed as previously described by Barrett and Kirschke (1981).

2.5. Kinetic constants

Kinetic constants of cathepsin L2 was determined according to Visessanguan, Benjakul, and An (2003), with a final concentration of Z-Phe-Arg-MCA ranging from 4.95 to 60 μmol/l.

2.6. Polyacrylamide gel electrophoreses

Analytical polyacrylamide gel electrophoresis (native-PAGE) was performed as described by Wang and Fan (2000) at pH 8.0 using 8% gel.

SDS–PAGE was conducted by the Laemmli's method (Laemmli, 1970). The stacking and resolving gels were 4% and 11% (7.5% for the analysis of myosin degradation), respectively. The samples were reduced with 10% β-ME at 100 °C for 5 min, while, for the non-reducing SDS–PAGE, this treatment was cancelled. Then 10 μl of sample was loaded on each lane and run on a Mini PROTEIN 3 apparatus acquired from Bio-Rad (BioRad Laboratories Inc., Richmond, CA) with a constant voltage of 120 V. After electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 40% ethanol and 10% acetic acid, and destained with 45% ethanol and 5% acetic acid.

The gelatin–substrate-active SDS–PAGE was carried out with 0.2% gelatin in 8.0% resolving gel, and without the treatments of adding β-ME and heating for the enzyme samples. The activity staining was performed as described by García-Carreño, Dimes, and Haard (1993) with a slight modification. The activity zone was developed in McIlvaine's Buffer of pH 5.5, containing 1 mmol/l DTT, at 37 °C for 9–12 h.