Food Chemistry 125 (2011) 1091–1096

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Identification of calpastatin, μ-calpain and m-calpain in Atlantic salmon (*Salmo salar* L.) muscle

M. Gaarder [a,*], M.S. Thomassen [a], E. Veiseth-Kent [b]

[a] Norwegian University of Life Sciences, Department of Animal and Aquacultural Sciences, Aborétveien 6, NO-1432 Ås, Norway
[b] Nofima Mat AS, Osloveien 1, NO-1430 Ås, Norway

ARTICLE INFO

Article history:
Received 18 May 2010
Accepted 28 September 2010

Keywords:
Atlantic salmon
Calpastatin
m-Calpain
Muscle
μ-Calpain

ABSTRACT

A soft fish muscle is generally considered as a poor quality trait among consumers and producers. This degradation and softening of *post mortem* muscle is thought to be partly caused by proteolytic enzymes such as the calpain system. Separation and identification of μ-calpain and m-calpain and their inhibitor – calpastatin, from Atlantic salmon (*Salmo salar*) muscle were for the first time assessed in this study. A two-step chromatography approach was used, starting with a hydrophobic interaction column and followed by an anion exchange column. Calpastatin was successfully separated from calpain by hydrophobic interaction chromatography, and following the anion exchange chromatography, two forms of calpastatin (I and II) and two forms of calpain (micro (μ) and milli (m)) were revealed. The proteolytic activity of μ-calpain was detectable with column chromatography, but not consistently detected with casein zymography, and m-calpain was detected with both chromatography and casein zymogram. The proteolytic activity of m-calpain per g muscle was 15 times higher than that of μ-calpain. μ-Calpain had a temperature optimum of 15 °C and a maximum calcium requirement at 0.2 mM, while m-calpain had temperature optimum at 25 °C and a maximum calcium requirement of 0.6 mM. The two forms of calpastatin differed in inhibitory activity with calpastatin II having the highest activity. Both calpastatins tolerated heat treatment, as previously seen for mammals, and they kept their activity when stored at −80 °C, but not at −20 °C. The calpain to calpastatin ratio was 1:4.5 as observed for beef muscle. This study provides evidence that two calpain isoforms, likely to be μ- and m-calpain, in addition to two forms of calpastatin exist in Atlantic salmon muscle.

1. Introduction

It is of great importance to increase the current knowledge regarding factors affecting the quality of muscle foods in order to produce a more consistent, high quality product. Several experiments have shown that tenderness in meat (Geesink, Kuchay, Chishti, & Koohmaraie, 2006; Koohmaraie, 1992; Koohmaraie & Geesink, 2006; Raser, Posner, & Wang, 1995; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2004) and the development of soft muscle in fish (Geesink, Morton, Kent, & Bickerstaffe, 2000; Ladrat, Michael, Verrez-Bagnis, Joëlle, & Joël, 2000) are partly caused by proteolytic activity. Two proteolytic systems, the calpain system and the lysosomal cathepsins, are known to hydrolyze myofibrillar proteins during post mortem storage of mammalian and fish muscle (Beltran et al., 1997; Chéret, Delbarre-Ladrat, de Lamballerie-Anton, & Verrez-Bagnis, 2007). Their relationship to textural changes in fish muscle is, however, still under debate.

The ubiquitous calpain system consists of μ-calpain, m-calpain and the endogenous inhibitor calpastatin. However, reports regarding calpains in fish muscle are not consistent when it comes to the findings of μ- and m-calpain (Ladrat, Verrez-Bagnis, Noel, & Fleurence, 2002; Saito, Li, Thompson, Kunisaki, & Goll, 2007; Salem, Kenny, Killefer, & Nath, 2004a). Calpastatin is the only known inhibitor specific for the calpains (Ciobanu et al., 2004; Goll, Thompson, Li, Wei, & Cong, 2003; Saito et al., 2007) and several studies have shown an inverse relationship between calpastatin activity and meat tenderness (Koohmaraie, 1992; Kristensen et al., 2002).

Information on the calpain system in fish is still scarce, even though this system is thought to be of importance for the stability of muscle texture. To the best of our knowledge, no studies have so far been conducted on the calpain system in Atlantic salmon (*Salmo salar*) muscle. Thus, the aim of the present study was to separate and detect calpain and calpastatin in Atlantic salmon muscle and to characterise their activities.

* Corresponding author. Tel.: +47 64 96 52 40; fax: +47 64 96 51 01.
E-mail addresses: mari.gaarder@gmail.com, mari.gaarder@umb.no (M. Gaarder).

0308-8146/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2010.09.095

2. Materials and methods

2.1. Materials

Acrylamide (99.9%) and bis-acrylamide were from BioRad Laboratories Inc., USA; casein hammersten, coomassie blue (R-250), ammonium persulphate (APS) and tetra-methyl-ethylenediamine (TEMED) from VWR International Ltd., England; 2-mercaptoethanol (MCE), diethylaminoethyl (DEAE) sephacel, leupeptin, trypsin inhibitor (Type I-P) and phenylmethylsulfonyl fluoride (PMSF) were from Sigma–Aldrich, Arkema Inc., St. Louis, MO.; phenyl-Sepharose was from GE Healthcare, England.; Brij 35 was from Merck Chemicals Ltd., England.

2.2. Muscle samples

Four Atlantic salmon used in this experiment were collected in May 2008 at Nofima Marine research station at Averøy, Norway, and only sexually immature salmon, with an average weight of 4 kg were used. After anesthetisation with MS222 (Pharmaq AS, Norway), the salmon were sacrificed with a blow to the head and bled. White muscle samples were taken from the dorsal musculature 5–10 min post mortem, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Extraction and hydrophobic interaction chromatography

Muscle samples from the four salmon were pooled and a sample of 400 g muscle were taken and homogenized with a PT 3100 Polytron (Kinematica Inc., Switzerland) in 1720 mL of extraction buffer I (20 mM Tris base, 5 mM EDTA, 0.05% MCE, pH 8.0) for 3 × 30 s. Inhibitors (100 mg mL^{-1} Trypsin inhibitor, 120 mg mL^{-1} Leupeptin and 2 mM PMSF (1.0% vol/vol)) were added to the buffer just before homogenisation. The homogenate was then centrifuged at 16,000×g_{max} for 1.5 h, 4 °C. After centrifugation the supernatant was filtered through glass wool (which had been prewashed) to remove excess fat and adjusted to 125 mM KCl by adding 2 M KCl. The supernatant (1575 mL) was then loaded onto a 5.0 × 13.0 cm phenyl-Sepharose column equilibrated with equilibration buffer A (125 mM KCl, 20 mM Tris base, 1 mM EDTA, 0.1% MCE, pH 7.5), and the column washed with equilibration buffer A (2 L) until the A_{278} outflow was less than 0.2 (for a schematic overview, see Fig. 1). Calpastatin passed through during sample loading and washing of the column. Calpain was then eluted (114 mL/h) with 1.6 L elution buffer A (1 mM EDTA, 0.1% Brij 35, 0.1% MCE, pH 7.5). Fractions containing calpastatin and calpain activity were localized with standard casein assay as described by Koohmaraie (1990).

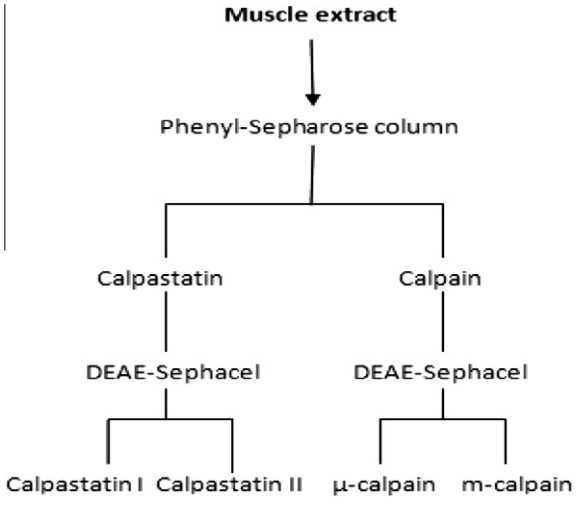

Fig. 1. A schematic diagram summarising the steps used to purify calpastatin I, calpastatin II, μ-calpain and m-calpain from Atlantic salmon muscle by the use of a two-step chromatographic column pathway.

Calpastatin-containing fractions were pooled and salted out with 80% $(NH_4)_2SO_4$ saturation, stirred over night at 4 °C; and then centrifuged at 16,000×g_{max}, for 1 h at 4 °C. The pellet was dissolved in buffer B (20 mM Tris base, 1 mM EDTA, 0.1% MCE, pH 7.5) and dialyzed over night at 4 °C against dialysis buffer (1 mM $KHCO_3$, 5 mM EDTA, 5 mM MCE, pH 7.5). After centrifugation (28,000×g_{max}, 1.5 h, 4 °C), the supernatant was filtered through glass wool.

2.4. Ion exchange chromatography

The pooled and salted out calpastatin fractions (290 mL) were loaded by gravity onto 5.0 × 17 cm column packed with DEAE-sephacel and equilibrated with buffer B. After loading, the column was washed with 600 mL of buffer B. The elution profile was a linear gradient from 0 to 100 mM KCl with 750 mL of each concentration, then 300 mL with 100 mM KCl and then a linear gradient from 100 to 300 mM KCl with 500 mL of each concentration (114 mL/h). Calpastatin was localized using standard casein assay as described by Koohmaraie (1990).

Fractions collected from the hydrophobic interaction column containing calpain were pooled (850 mL) and then loaded onto a 2.6 × 25 cm column packed with DEAE-sephacel and equilibrated with buffer C (20 mM Tris base, 1 mM EDTA, 0.1% MCE, pH 6.5). The column was washed with 150 mL of buffer D (20 mM KCl, 20 mM Tris base, 1 mM EDTA, 0.1% MCE, pH 6.5), and then eluted (58.9 mL/h) with a linear gradient from 20 to 300 mM KCl followed by 75 mL of 300 mM KCl. Fractions containing calpain activity were localized using standard casein assay as described by Koohmaraie (1990). Fractions from different peaks with calpain activity were pooled separately and dialyzed against buffer E (40 mM Tris base, 0.5 mM EDTA, 0.05% MCE, pH 7.35) over night and then centrifuged for 45 min at 16,000×g_{max}, 4 °C. Calpain activity in samples from the two peaks was determined in triplicates by Bodipy-FL assay as described by Thompson, Saldaña, Cong, and Goll (2000).

2.5. Characterisation of calpains

Both calpain peaks were tested for calcium requirement and temperature stability. Calcium requirement for the first calpain peak (calpain I) was tested with standard casein assay (Koohmaraie, 1990) in the presence of 0.1–0.5 mM $CaCl_2$. The same assay was used for the second calpain peak (calpain II), but in the presence of 0.25–3 mM $CaCl_2$. Both calpain activities were measured in triplicates.

Activity of the two calpains was also measured in triplicates at different temperatures; 4 °C, 15 °C, 25 °C and 36 °C, using the same assay as described above.

2.6. Stability of calpastatin

Pooled calpastatin fractions were frozen (−80 °C and −20 °C) for two weeks or stored in fridge (4 °C) for two weeks. Samples stored at 4 °C were further heated (50 °C, 73 °C and 96 °C) in water bath for 15 min. After heating, samples were kept on ice for 15 min and then centrifuged for 15 min at 13,000 rpm at 4 °C. Activity of calpastatin I and II were measured in triplicates after treatment at different temperatures. The calpastatin activity remaining after the different temperature treatments were analyzed in triplicates with Bodipy-FL assay as described by Thompson et al. (2000),