Food Chemistry 148 (2014) 1–6

Contents lists available at ScienceDirect

Food Chemistry

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

Cleavage of the calpain inhibitor, calpastatin, during postmortem ageing of beef skeletal muscle

Feng Huang [a], Ming Huang [b], Hong Zhang [a], Bing Guo [b], Dequan Zhang [a], Guanghong Zhou [b,*]

[a] Institute of Agro-Products Processing Science and Technology, Chinese Academy of Agricultural Science, Beijing 100193, China
[b] Key Laboratory of Meat Processing and Quality Control, Ministry of Education China, College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

ARTICLE INFO

Article history:
Received 23 May 2013
Received in revised form 5 August 2013
Accepted 2 October 2013
Available online 10 October 2013

Keywords:
Calpastatin
Caspase
Calpain
Beef muscle
Ageing

ABSTRACT

The objective of this study was to investigate the contribution of caspase and calpain, on the proteolysis of calpastatin in postmortem beef muscle, by examining the influences of calpain inhibitor MDL-28170 and caspase-3 inhibitor DEVD-CHO on calpastatin degradation and the *in vitro* proteolysis of calpastatin by caspase-3, -6 and μ-calpain. In this study, both calpain- and caspase-3-inhibitors suppressed postmortem degradation of calpastatin. *In vitro* treatment of calpastatin with μ-calpain resulted in degradation products similar in size to those occurring naturally in aged beef muscle. With addition of caspase-3, only the 100 kDa degradation fragment was present during the early phase of ageing, and subsequently, was likely to have been inactivated by calpain or other factors. Therefore, calpain was the major contributor to the proteolysis of calpastatin in postmortem beef muscle. While caspase-3 was involved in calpastatin degradation during the early postmortem period, calpastatin maybe plays an important role in bridging the gap between caspase and calpain systems.

1. Introduction

Calpain is widely considered to represent the major protease activity in the postmortem (PM) proteolysis of myofibrillar proteins leading to meat tenderization (Huff-Lonergan, Zhang, & Lonergan, 2010; Koohmaraie & Geesink, 2006). With this background, calpastatin, a highly specific endogenous inhibitor of calpain, also receives much attention with regards to the tenderization of meat PM. So far, several studies have demonstrated that calpastatin activity decreases gradually during PM ageing (Boehm, Kendall, Thompson, & Goll, 1998), and the change accounts for a significant amount of the variation in beef tenderness (~40%), more than any other single measure (Shackelford et al., 1994). Therefore, it is important to understand the mechanism of calpastatin degradation in PM muscle. Although some studies have demonstrated that the *in vitro* degradation pattern of calpastatin by calpain is similar to that observed in naturally aged meat, the degradation does not lead to the complete loss of inhibitory activity of calpastatin, and even after extensive proteolysis by calpain, most of the inhibitory activity remains (Doumit & Koohmaraie, 1999). Furthermore, the question of how calpain can be activated in PM muscle when the muscle contains an excess of calpastatin is also unclear (Boehm et al., 1998). It is therefore possible that, in addition to calpain, some other endogenous proteolytic enzymes may be activated before calpain and therefore take part in the degradation or inactivation of calpastatin. The caspase system has become a new focus of attention in the field of meat ageing since it was first proposed that it was a likely candidate for PM tenderization (Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006). There is currently compelling evidence which demonstrates that caspases can be activated during normal PM ageing and take part in the PM tenderization process (Chen et al., 2011; Huang, Huang, Xu, & Zhou, 2009; Kemp & Parr, 2008; Kemp, Parr, Bardsley, & Buttery, 2006), although there is some dispute (Mohrhauser, Underwood, & Weaver, 2011; Underwood, Means, & Du, 2008). It has also been suggested that caspases possibly function in PM tenderization of meat through calpastatin degradation (Kemp, King, Shackelford, Wheeler, & Koohmaraie, 2009). In addition, it has been demonstrated that calpain and caspase interact through calpastatin in many apoptotic models (Han, Shirasaki, & Fukunaga, 2006; Wei et al., 2005). Because PM muscle cells also die through apoptosis (Becila et al., 2010), and so calpastatin may be a link factor between caspase and calpain. Therefore, the objective of this study was to investigate the contribution of caspase and calpain to the proteolysis of calpastatin in postmortem beef muscle.

2. Materials and methods

2.1. Sampling and treatment

Three 2.5 years old crossbred cattle (Luxi × Simmental bulls with live weight 450 ± 50 kg) were slaughtered humanely at a commercial meat processing company (HanSen Meat Co. Ltd,

* Corresponding author. Tel.: +86 25 84395730; fax: +86 25 84395939.
E-mail addresses: ghzhou@njau.edu.cn, zhou863@126.com (G. Zhou).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.10.016

Anhui, China) according to the requirements of National Standards of PR China "Operating Procedures of Cattle Slaughter". After animal exsanguination, *Longissimus thoracis* (LT) muscles (from 12th thoracic vertebrae to 5th lumbar vertebrae) were excised from the right side of carcasses within 30 min. Subsequently, approximately 30 g muscle was frozen rapidly in liquid nitrogen as 0 d samples; another 30 g muscle was aged 6, 12, 24, and 72 h, which served as normal ageing samples; and lastly, approximately 30 g muscle was dissected into small pieces (ca. 0.2 g/piece). These minced muscles were subdivided into four fractions and soaked in one of the following four buffers in the ratio 1:1 (w/v) (meat/buffer), respectively: (1) 100 mM NaCl and 2 mM NaN_3 (control); (2) control + 100 μM MDL-28170 (MDL) (Calbiochem, 208722); (3) control + 100 μM DEVD-CHO (DEVD) (Sigma), and kept for 6, 24, or 72 h at 4 °C. At the end of each storage period, the samples were taken individually and treated as were 0 d samples.

2.2. Calpastatin preparation

Calpastatin was extracted according to the procedure as previously described (Shackelford et al., 1994) with minor modifications. Briefly, muscle samples were homogenised in approximately 3 volumes of extraction buffer containing 10 mM EDTA, 0.1% (v/v) β-mercaptoethanol (MCE), protease inhibitor cocktail (Roche) and 100 mM Tris–HCl, pH 8.3 for 15 s two times at a speed of 15,000 rpm with a 30 s rest between each burst. The homogenate was centrifuged at 35,000*g* for 20 min. The resultant supernatant was heated in a water bath (preheated to 95 °C) for 15 min to denature calpains. Following heating, samples were chilled in an ice water bath and the coagulated protein was dispersed with a small glass rod. Then the samples were centrifuged at 35,000*g* for 30 min and the resultant supernatant was filtered through cheesecloth. The supernatant was precipitated with 15% (w/v) trichloroacetic acid (TCA) (Geesink, Nonneman, & Koohmaraie, 1998) to increase the concentration of calpastatin. These samples were used to detected *in vivo* degradation of calpastatin. For the *in vitro* digestion of calpastatin the procedure was modified as follows: after the second centrifugation, the samples were not TCA-precipitated but were concentrated by lyophilisation. Protein concentration was determined by the Bradford assay (Bio-Rad).

2.3. Sarcoplasmic protein extraction

Extraction of sarcoplasmic proteins was performed according to Chen et al. (2011) with slight modification. The minced muscle was homogenised in 3 volumes of precooled extraction buffer containing 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 5 mM sodium fluoride, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (10 ml/tablet). The homogenate was centrifuged at 15,000*g* for 20 min at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad).

2.4. In vitro digestion of calpastatin

Three hundred micrograms of heat-soluble calpastatin prepared from time 0 samples were incubated in the incubation buffer (IB) containing 50 mM HEPES, 10 mM DTT, 10% sucrose, 0.1% CHAPS, and 5 mM EDTA, pH 7.2 (designated as control) or the buffer containing 10 units of recombinant caspase-3 (Bioversion, CA, USA) or 10 units of recombinant caspase-6 (Bioversion, CA, USA), where 1 unit is defined as the amount of enzyme that cleaves 1 nM DEVD-pNA for caspase-3 and 1 nM VEID-pNA for caspase-6, respectively, per hour at 37 °C. After 2 or 12 h of incubation at 30 °C, the samples were denatured at 100 °C for 5 min to stop the reaction.

2.5. SDS–PAGE and immunoblotting

All resultant samples for SDS–PAGE and immunoblotting were well mixed with sampling treatment buffer (125 mM Tris, 4% SDS, 20% glycerol, pH 6.8). The mixture was heated in a 50 °C water bath for 20 min, and then stored at −80 °C until loading.

The acrylamide percentage varied depending on the protein of interest: For calpastatin, 10% gels were used and for caspase-3, 12.5% gels were used. A 4.5% polyacrylamide gel was used for stacking gel. The composition of the gels was as described in our previous paper (Huang, Huang, Ma, Xu, & Zhou, 2012). After electrophoresis, the protein of interest was transferred onto polyvinylidine fluoride membranes (Millipore) using a wet transfer apparatus (BioRad Laboratories). The membranes were blocked with a blocking buffer (5% nonfat dry milk, 0.05% Tween 20, 137 mM NaCl, 5 mM KCl and 20 mM Tris–HCl, pH 7.4) for 90 min at room temperature. After blocking, the membranes were exposed to one of the following primary antibodies for 16 h: rabbit anti-caspase-3 polyclonal antibody (Calbiochem), or mouse anti-calpastatin monoclonal antibody (Thermo). Subsequently, blots were exposed to the reciprocal secondary antibody. Immunoreactive protein bands were detected by enhanced chemiluminescence (Thermo).

2.6. Determination of caspase-3 activity

Caspase-3 activity was determined by using a Caspase-3 Fluorometric Assay Kit (PharMingen). Sarcoplasmic proteins were added to the reaction buffer consisting of 20 mM HEPES, pH 7.4, 10% glycerol, and 2 mM DTT, and the mixture was pre-incubated for 30 min at 37 °C. The reaction was started by addition of the caspase-3 fluorogenic substrate Ac-DEVD-AMC. After incubation for 1 h at 37 °C, the fluorescence was measured using excitation and emission wavelengths of 360 and 460 nm, respectively. Results were expressed as percentage of fluorogenic units in different PM time samples versus those in time 0 d samples.

2.7. Statistical analyses

The data from all the determination were analysed by one-way analysis of variance using SAS statistical software (Version 8.2, SAS Inst.), and differences among mean values were determined by the least significant difference comparison procedure with a significance level of $P < 0.05$.

3. Results

3.1. Postmortem degradation of calpastatin in beef muscle

When beef skeletal muscle was taken immediately after slaughter, only intact calpastatin was evident, as indicated by labelling with monoclonal anti-calpastatin antibody in the region of ca. 130 kDa (Fig. 1). This is consistent with the results reported previously (Doumit & Koohmaraie, 1999; Geesink et al., 1998). During short-term storage of beef muscle, intact calpastatin was gradually degraded and disappeared completely at 3 days PM, as determined by Western blotting (Fig. 1). The loss of intact calpastatin initially resulted in the production of two calpastatin degradation fragments, which were about 100 and 70 kDa in size. As the 100 kDa fragment formed during the initial storage period, it was further degraded to a fragment of size 70 kDa (Fig. 1).