Food Chemistry 200 (2016) 315–321

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Identification of different domains of calpain and calpastatin from chicken blood and their role in post-mortem aging of meat during holding at refrigeration temperatures

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ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form 19 December 2015
Accepted 8 January 2016
Available online 11 January 2016

Keywords:
Calpains
Calpastatin
Zymography
Proteolysis
Muscle
Blood

ABSTRACT

The aim of this study was to develop a simple, specific and rapid analytical method for accurate identification of calpain and calpastatin from chicken blood and muscle samples. The method is based on liquid–liquid extraction technique followed by casein Zymography detection. The target compounds were extracted from blood and meat samples by tris buffer, and purified and separated on anion exchange chromatography. It has been observed that buffer (pH 6.7) containing 50 mM tris-base appears to be excellent extractant as activity of analytes was maximum for all samples. The concentrations of μ-, *m*-calpain and calpastatin detected in the extracts of blood, breast and thigh samples were 0.28–0.55, 1.91–2.05 and 1.38–1.52 Unit/g, respectively. For robustness, the analytical method was applied to determine the activity of calpains (μ and *m*) in eighty postmortem muscle samples. It has been observed that μ-calpain activity in breast and thigh muscles declined very rapidly at 48 h and 24 h, respectively while activity of *m*-calpain remained stable. Shear force values were also declined with the increase of post-mortem aging showing the presence of ample tenderness of breast and thigh muscles. Finally, it is concluded that the method standardized for the detection of calpain and calpastatin has the potential to be applied to identify post-mortem aging of chicken meat samples.

1. Introduction

Tenderness is one of the most single important factors affecting the eating quality of meat. Many studies revealed that the meat tenderization process is a complex mechanism, and can be affected by several pathways including pre- and post-slaughter factors and their interactions. The tenderness of meat is determined by the extent of proteolysis, which is presumably due to the action of calpains, calcium-dependent endogenous proteases (Koohmaraie, 1996). Calpastatin is another endogenous enzyme inhibitory to calpains, is also widely distributed in the muscles. Both these enzymes are also present in the blood. But the enzymatic concentrations present in blood, same may not be distributed in the muscles for their activity due to changes in pH, ionic strength, temperature etc. It seems that calpains are proteolytic enzymes that cause post-mortem proteolysis and tenderness, but it is not still clear whether μ-calpain alone is responsible or both the μ- and *m*-calpains are involved in the process since μ-calpain and calpastatin gradually lost their activity with the post-mortem aging but *m*-calpain remained stable (Vidalenc, Cottin, Merdaci, & Ducastaing, 1983; Wheeler, Shackelford, & Koohmaraie, 2000). Whatsoever the facts, by determining the enzymatic concentrations in blood from live birds, it is possible to predict and manage tenderness of meat by changing aging time for maximizing consumer quality perception.

Diverse ranges of buffers with varying salt concentrations were used for extraction and separation of μ- and *m*-calpains and calpastatin from biological samples (Geesink & Koohmaraie, 1999a; Huang, Huang, Ma, Xu, & Zhou, 2012; Koohmaraie, Seidemann, Schollmeyer, Dutson, & Crouse, 1987; Kretchmar, Hathaway, Epley, & Dayton, 1990), but a very few studies were carried out to understand the influence of buffer on the extraction efficiency of these enzymes. Further, to best of our knowledge, there is no published literature on the simultaneous extraction of μ- and *m*-calpains and calpastatin from two different matrices i.e., blood and tissue samples, since blood sample requires additional pre-treatment and purification steps for their proper identification.

Another area of concern is the visualization of μ-calpain in *in vitro* casein Zymography. As μ-calpain is calcium dependent protease and requires micro-molar (μM) level of calcium for its activity, work relating to optimization of $CaCl_2$ concentration in

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http://dx.doi.org/10.1016/j.foodchem.2016.01.031
0308-8146/

proteolytic buffer is still pending. So, this study is also focused on the standardization of proteolytic buffer for maximum visualization of μ-calpain from blood and tissue samples. The standardized analytical method was applied to extract calpains and calpastatin from chicken breast and thigh muscles to elucidate their activity in post-mortem muscle during holding at 4 ± 1 °C. The changes in muscle pH and Warner–Bratzler (W–B) shear force values were also monitored to determine optimum aging time for spent chicken meat.

2. Materials and methods

2.1. Chemicals and reagents

Casein from bovine milk purified powder, molecular weight cut-off filter (12 kDa), DEAE-Sephacel (anion exchanger) and protease inhibitors (leupeptin hemisulphate, ovomucoid, phenylmethane sulphonyl fluoride) were procured from Sigma–Aldrich, St. Louis, MO, USA. Novagen perfect protein marker (10–225 kDa) was obtained from Merck Millipore Ltd., Mumbai, India. Econo-Column (1.5 × 8.5 cm) used for anion exchange chromatography was procured from Bio-Rad Laboratories, Lucknow, India. All other solvents and reagents required for this experiment were of standard quality and procured from s.d. Fine Chemicals, New Delhi, Sisco Research Laboratories, Mumbai and Merck Specialist Pvt. Ltd., Mumbai, India.

2.2. Sample collection

A total 80 muscle samples (White Leghorn spent hen, 56 wk of age) comprised of each of 40 breast and thigh muscles were collected from Experimental Poultry Processing Plant of ICAR-Central Avian Research Institute, Izatnagar, Bareilly, India. The samples were collected from grading table where tissue samples were excised, packed and chilled at 0–1 °C immediately. About 100 g of muscle samples were collected and transferred into a self-sealing LDPE bags. The bags were labeled, chilled, and finally, transferred to the laboratory under chilled (0–1 °C) condition for analysis. Blood samples (15 mL from each of 40 birds) were collected during bleeding operations. The samples were collected in vials containing ethylenediaminetetraacetic acid (1 mg/mL blood) and transferred to the laboratory for processing immediately.

2.3. Preparation sample extract

For preparation of sample extract, about 1 g of blood was homogenized with 3 volumes of ice-cooled extraction buffer containing 20 mM tris-base (pH 5.9), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.05% (v/v) 2-mercaptoethanol (MCE) and 0.1% (v/v) Triton X-100. To avoid functioning of unwanted enzymes, protease inhibitors [2 mM PMSF, 100 mg/L ovomucoid, 6 mg/L leupeptin] were added just before the extraction. The extract was then centrifuged at 20,000×*g* for 20 min at 4 °C (Make-Eppendorf 5427R, Hamburg, Germany) and supernatant was decanted in a separate centrifuge tube. The collected supernatant was centrifuged once again and then filtered, and finally, collected in another centrifuge tube (Raser, Posner, & Wang, 1995; Veiseth & Koohmaraie, 2001). Other two extracts using 50 mM (pH 6.7) and 100 mM (pH 8.3) tris-base containing buffers were also prepared in similar manner.

However, in preparation of sample extracts for muscle samples, they were first trimmed-off for removal of excessive connective tissues, fat and fascia, and then cut into fine pieces. About 3 g of finely cut samples were homogenized with 6 volumes of extraction buffer containing 20 mM tris-base (pH 5.9), 10 mM EDTA and 0.05% (v/v) MCE. Other processing steps were similar to that of preparation of sample extracts for blood samples.

2.4. Purification and separation of μ- and m-calpains and calpastatin from sample extract

Sample extracts obtained earlier were purified using dialysis tube of 12 kDa MWCO cellulose filter (Sigma–Aldrich) for overnight at 4 ± 1 °C. The ratio of dialysis buffer (pH 7.4) [40 mM tris-base, 5 mM EDTA and 0.05% (v/v) MCE] and sample extract was maintained at 20:1. Fresh dialysis buffer was prepared at each time and that was stored at 4 ± 1 °C until use.

For separation μ- and *m*-calpains as well as calpastatin, anion exchange column chromatography was performed, in which supernatant obtained after dialysis was loaded on a previously equilibrated column containing 5 cm settled DEAE-Sephacel matrix. The column was equilibrated by three washings (3 × 20 mL) using equilibrium buffer (pH 7.4) comprising of 40 mM tris-base, 0.5 mM EDTA and 0.05% (v/v) MCE. The sample extract corresponding to 1 g of blood or 3 g of muscle was loaded on the column, and then compounds were eluted using varying strength of NaCl buffer. Calpastatin was eluted at 100 mM NaCl while μ- and *m*-calpains were eluted at 200 mM and 400 mM NaCl concentrations, respectively (Geesink & Koohmaraie, 1999b; Karlsson, Gustavsson, Hall, & Nilsson, 1985). A total 12 mL fractions were collected (4 mL each) using each eluting buffer and were stored at −20 °C for analysis.

2.5. Analysis

2.5.1. Casein zymography

Casein zymography method is based on the principle that casein molecules present in the zymogram gel are catalyzed by the calpains in the presence of Ca^{2+} ion in the solution (Huang et al., 2012). In resolving gel, casein (0.21%, w/v) was copolymerized with 10% (w/v) acrylamide and 0.4% (w/v) bis-acrylamide in 375 mM tris-base (pH 8.8). The stacking gel contained 4% (w/v) acrylamide and 0.16% (w/v) bis-acrylamide in 330 mM tris-base (pH 6.8) and was polymerized with 0.05% (w/v) ammonium persulfate (APS) and 0.01% (v/v) N′N′N′N′-tetramethylethylenediamine (TEMED). Polymerization of resolving gel was catalyzed by 0.04% (w/v) APS and 0.028% (v/v) TEMED. The casein gels were pre-run on a Mini-Protean tetra gel system (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 15 min at 4 °C with running buffer (pH 8.3) containing 192 mM glycine, 25 mM tris-base, 1 mM ethyleneglycol O-O′-bis (2-aminoethyl) N′N′N′N′-tetraacetic acid (EGTA), 1 mM EDTA, and 0.05% (v/v) MCE. Then crude extracts were loaded with sample buffer (3:1, v/v) [150 mM tris-base (pH 6.8), 20% (v/v) glycerol, 0.75% (v/v) MCE and 0.04% (w/v) bromophenol blue] and run again for electrophoresis at 100 V for 4 h at 4 °C. At the end, the gels were taken out and incubated in proteolytic buffer (pH 7.4) [comprising of 20 mM tris-base, 10 mM dithiothreitol (DTT)] for 18–24 h at 20 °C. Three different proteolytic buffers were prepared using varying concentrations of $CaCl_2$ i.e., 1, 3 and 4 mM. In the region of presence of activated calpain, the casein is digested into small fragments that diffuse out of the gel. The gels were stained for 30–60 min with 40% methanol, 10% acetic acid and 0.25% (w/w) Coomassie Brilliant Blue G-250 and then kept overnight in de-staining solution containing 30% (v/v) methanol, 10% (v/v) acetic acid which gives clear bands indicating presence of calpains. Another gel was also maintained using non-calcium containing buffer (1 mM EGTA) and absence of band indicating calcium is required for activation of calpains to cause lysis of casein.

2.5.2. SDS–PAGE analysis

For identification of calpains and calpastatins in different purified fractions, SDS–PAGE was performed, in which, resolving gel